Pathogenesis of Cardiovascular Disease in the Presence and Absence of Rheumatoid Arthritis

Samuel Arion Curran BSc (Hons)

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Author’s Declaration

I declare that the work presented in this thesis is my own unless specifically stated otherwise.

Samuel Arion Curran

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Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory disease that although primarily affecting the joints is recognised as a systemic disease with a variety of co-morbidities. One such co-morbidity is cardiovascular disease (CVD) and patients with RA are at a two-fold increased risk of developing CVD compared to the general population. Indeed, CVD, principally as a result of atherosclerosis, is recognised as the leading cause of death in RA patients. In this study the vascular adventitia and serum samples from patients with co-existing RA and CVD were investigated to identify factors that could explain the increased CVD morbidity observed in RA patients. This involved culture-independent identification of bacterial signatures and histological evaluation for pro-inflammatory molecules and potential RA-associated autoantigens in the aortic adventitia. Additionally, serum samples from healthy controls, patients with RA or CVD alone, and patients with co-existing RA and CVD, were screened for a broad range of cytokines, chemokines and growth factors. These data provided the basis for further studies into the effect of cytokines on foam cell formation.

Histological evaluation of the aortic adventitia revealed that pronounced inflammatory infiltrates were detectable in all patients with CVD, regardless of rheumatic disease. To assess whether or not the inflammatory composition of the aortic adventitia differed between patients with or without RA, sections were examined using immunohistochemistry for a markers including TNFα, CD21 and heat shock proteins (HSP) 47 and 60, both of which have been implicated in the pathogenesis of RA and CVD. TNFα and HSP47 were confirmed in the aortic adventitia; however, neither CD21 nor HSP60 were detected. The expression of total TNFα was significantly elevated in RA+CVD compared to non-RA CVD patients. These data demonstrate that the aortic adventitia is a site of considerable immunological function and suggest a possible role for TNFα overexpression in the pathogenesis of CVD in RA.

Bacterial infection has been implicated as a causative agent for both RA and CVD. The present study used culture-independent methods to identify bacteria in the aortic adventitia of RA+CVD and non-RA CVD patients. The presence of bacterial DNA was confirmed in three of 11 RA+CVD and four of 11 non-RA CVD patients. The bacterial flora of RA+CVD patients was significantly less heterogeneous compared to non-RA CVD patients. *Methylobacterium oryzae* was detected in every RA+CVD patient positive for the presence of bacterial DNA. Subsequent in *vitro* characterisation demonstrated that *M.*

oryzae stimulates mild Toll-like receptor 2 (TLR2), but not TLR4, signalling and, upon challenge of primary human macrophages, produces a robust pro-inflammatory response. Importantly, these findings implicate *M. oryzae* infection as playing a potential role in the pathogenesis of CVD in RA patients.

Previous studies have argued that the persistently high levels of systemic inflammation exhibited by RA patients are critical to elevated CVD risk. In the current study it was demonstrated that RA patients exhibit an altered systemic immune profile compared to non-RA CVD patients and healthy controls. The pro-inflammatory phenotype in co-morbind CVD and RA is suggestive of an environment that may promote atherosclerosis. Furthermore, RA+CVD patients were shown to possess an altered systemic immune phenotype compared to both RA and CVD patients. Notably, IL-1β, IL-2, IL-5, IL-8, IL-13, IL-17, Monokine induced by gamma interferon (MIG), Granulocyte colony-stimulating factor (G-CSF) and Granulocyte macrophage colony stimulating factor (GM-CSF) expression was greater in the serum of RA+CVD patients compared to the additive values of the RA and CVD patients.

The formation of lipid-rich foam cells is a major feature of atherosclerosis pathogenesis and is partially dependent on the inflammatory environment. The present study demonstrated that GM-CSF stimulation significantly decreased the rate at which human primary macrophages could endocytose oxLDL and form foam cells. The effect of dextran sulphate, a known competitive inhibitor of scavenger receptors, on the ability of GM-CSF stimulated macrophages to differentiate into foam cells revealed that GM-CSF decreases the concentration of dextran sulphate required to successfully inhibit scavenger receptor-mediated ox-LDL uptake; suggesting that GM-CSF-stimulated macrophages express less scavenger receptors on the cell surface. However, subsequent investigation demonstrated that GM-CSF stimulation did not decrease expression of the currently recognised scavenger receptors that are capable of oxLDL recognition (SR-A1, SR-B1, CD36 and MARCO). These data imply that the overexpression of GM-CSF observed in RA and RA+CVD patients may provide some atheroprotective benefit. This has clinical implications for future anti-RA drugs which target GM-CSF function.

In summary, the data presented in this thesis demonstrate that RA+CVD patients exhibit immunological and pathological alterations both in the aortic adventitia and systemically. Further research will, over time, provide insight into the mechanisms underlying increased CVD burden in RA.

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Table of Contents

List of Tables

List of Figures

Abbreviations

ACR	**American College of Rheumatology**
ACPA	**Anti citrullinated peptide antibody**
APC	**Antigen presenting cell**
ApoE$^{-/-}$	**Apolipoprotein E knock out mouse**
BMI	**Body mass index**
CABG	**Coronary artery bypass graft**
CCP	**Cyclic citrullinated peptide**
$CuSo_4$	**Copper sulphate**
CVD	**Cardiovascular disease**
CRP	**C-reactive protein**
DM	**Diabetes mellitus**
EDTA	**Ethylene diamine tetracetic acid**
ELISA	**Enzyme-linked immunosorbent assay**
FACS	**Fluorescent Activated Cell Sorter**
HDL	**High density lipoprotein**
HRP	**Horseradish peroxidase**
HS	**Healthy samples**
Ig	**Immunoglobulin**
II	**Inflammatory infiltrate**
IMA	**Internal mammary artery**
LDL	**Low density lipoprotein**
LOS	**Lipooligosaccharide**
LPS	**Lipopolysaccharide**
NA	**Not available**
NTC	**No template control**
oxLDL	**Oxidized low density lipoprotein**
PAMP	**Pathogen associated molecular pattern**
PBMCs	**Peripheral blood mononuclear cells**

PCR	**Polymerase chain reaction**
PBS	**Phosphate buffered saline**
PRR	**Pattern recognition receptor**
PsA	**Psoriatic arthritis**
RA	**Rheumatoid arthritis**
RF	**Rheumatoid factor**
RPE	**R Phycoerythrin**
RPMI	**A cell culture medium discovered at ‘Roswell Park Memorial Institute’**
RPM	**Revolutions per minute**
SD	**Standard deviation**
SDS	**Sodium dodecyl sulphate**
SE	**Standard error**
SLE	**Systemic lupus erythematosus**
SMC	**Smooth muscle cell**
SR	**Scavenger receptor**
Th1	**T helper lymphocyte type 1**
Th2	**T helper lymphocyte type 2**
TLR	**Toll-like receptor**

1 Introduction

1.1 Overview

The term atherosclerosis describes a chronic inflammatory disorder of the arterial blood vessel wall in which immune cell activation and inflammation leads to fibrotic plaque formation, followed by destabilisation and rupture (Hansson, 2001). Risk of cardiovascular disease (CVD), as a result of atherosclerosis, is increased in patients diagnosed with inflammatory rheumatic disease such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (van Leuven et al., 2006). Indeed, CVD is the leading cause of mortality in both RA and SLE patients.

The research undertaken for this thesis explores the hypothesis that the association between CVD and inflammatory rheumatic disease may be explained by the chronic inflammatory state itself. Furthermore, several alternative hypotheses are explored in an effort to improve our understanding of why the association between CVD and RA arises. Enhanced understanding will enable the design and implementation of specific therapeutic approaches for patients with these diseases.

The following introduction will initially provide a brief overview of immunity before focusing in detail on RA, CVD and reviewing current popular thinking of why the association between these diseases exists.

1.2 Introduction to immunity

We are constantly exposed to pathogenic microbes that are swallowed, inhaled or inhabit mucous membranes or the skin. Whether these microbes cause disease or not is a result of both the microbes' pathogenicity and the integrity of the host defence mechanisms. The immune system comprises an interactive network of lymphoid organs, cells, growth factors, chemokines and cytokines (Parkin and Cohen, 2001). An effective immune system is essential to human health; however, in both RA and CVD the immune system attacks the body (self) leading to joint destruction and blood vessel inflammation.

Host immunity can be divided into two functionally distinct but related systems, termed the innate and the adaptive immune responses. These two systems have evolved to protect the host from the invasion of microbes (bacteria, viruses, protozoa and fungi), harmful substances and to eliminate modified or altered self.

The term innate immunity is occasionally used to describe physical barriers, but more commonly encompasses the elements of the immune system (macrophages, monocytes, neutrophils, cytokines, complement and acute phase proteins) that provide an immediate first line of defence against pathogenic challenge (Kumar et al., 2011). Although not antigen specific, the innate immune system is effectively able to discriminate foreign molecules from self (self tolerance).

The highly conserved nature of the innate immune response, which is present in even the simplest of animals, demonstrates its importance to host survival (Janeway and Medzhitov, 2002). The innate immune system initially exhibits effector mechanisms over invading pathogens through immediate activation of phagocytic antigen-presenting cells (APCs) and the complement pathway, and secretion of antimicrobial peptides. The simultaneous secretion of down-regulatory mediators, for example the anti-inflammatory cytokine IL-10, alleviates the risk of host tissue destruction arising from an exaggerated rapid inflammatory response.

Activation of the innate immune system is dependent upon recruitment and activation of phagocytes bearing pattern-recognition receptors (PRRs) (*Section 1.2.2*), with lectin-like activity. PRRs recognise foreign structures termed pathogen-associated molecular patterns (PAMPs) (*Section 1.2.1*) present on microbes, but not self cells (Medzhitov and Janeway, 2000). PRRs support recognition, uptake and phagocytosis by antigen-presenting cells. Furthermore, the innate immune system also utilises the complement system during recognition and removal of pathogens (*Section 1.2.3*).

The innate and acquired immune systems are not isolated systems but, through the secretion of soluble mediators and cellular contacts, are constantly interacting with each other. The cytokine and chemokine profile established during the innate immune response initiates the migration of antigen-specific T cells to the lymph nodes where they come into contact with APCs. Acquired immunity is dependent upon the innate system providing effective antigen presentation through class I and class II MHC antigens, which are predominantly located on the surface of macrophages and dendritic cells. Additionally, T helper (T_H) cells bind to MHC antigens and stimulate B cells to generate immunity by undergoing immunoglobulin (Ig) class switching and somatic hypermutation, resulting in the production of antigen specific antibodies.

Unlike the innate immune response, which is rapid and lacks specificity, the acquired immune response is highly precise but takes days to weeks to develop. The acquired

immune response exhibits memory so that subsequent exposure results in a more rapid and vigorous response.

The following sections will consider the immune system in more detail. In keeping with the research outlined in this thesis, particular attention will be paid to PRRs, their corresponding PAMPs and inflammation.

1.2.1 Pathogen-associated molecular patterns

Pathogen associated molecular patterns (PAMPs) are molecules associated with groups of microbes that are recognised and targeted by the innate immune system. Recent studies have suggested that chronic bacterial and viral infections may play a pivotal role in the pathogenesis of both CVD and RA (Haraszthy et al., 2000, Jacob et al., 1992, Alvarez-Lafuente et al., 2005). In chapter 4 of this thesis we investigate the potential role bacteria may play in the vasculature of RA and CVD patients.

PAMPs are classically defined as evolutionarily conserved pathogen-derived molecules that distinguish hosts from pathogens (Ausubel, 2005). PAMPs include lipopolysaccahride (LPS), bacterial flagellin, peptidoglycan and mannans. It is worth noting that non-pathogens also synthesise PAMPs and the term 'pathogen-associated' is a misnomer. A more accurate term would be 'microbe-associated molecular pattern' (Ausubel, 2005).

LPS is an essential constituent of the outer membrane of Gram-negative bacteria, and is often considered the prototypical PAMP (Holst et al., 1996, Rietschel et al., 1994). LPS typically consists of a hydrophobic domain known as lipid A (endotoxin), a nonrepeating core oligosaccharide and a distal polysaccharide (O-antigen) (Raetz and Whitfield, 2002). Lipid A, the hydrophobic anchor of LPS, makes up the outermost monolayer of most Gram-negative bacteria. The lipid A domain is highly conserved throughout Gram-negative bacteria and is essential to their viability (Brabetz et al., 1997). In humans, LPS is primarily targeted by phagocytic cells of the innate immune system (macrophages, monocytes, neutrophils, dendritic cells). TLR4 in association with MD-2 is responsible for the physiological recognition of LPS (*see Section 1.2.2*) (Medzhitov et al., 1997, Shimazu et al., 1999).

Lipid A activation of TLR4 triggers the biosynthesis of pro-inflammatory cytokines such as TNFα and IL-1β (Beutler and Cerami, 1988, Dinarello, 1991), and activates the

expression of co-stimulatory molecules required for the adaptive immune response (Medzhitov and Janeway, 2000). LPS presence also induces an elevated expression of IFNγ by macrophages which acts to further enhance the sensitivity and magnitude of monocytes immune response to LPS (Fultz et al., 1993). Furthermore, IFNγ induces increased TLR4 expression in human monocytes and several pro-inflammatory cytokines (Muzio et al., 2000). LPS injections in apolipoprotein E (apoE)-deficient mice (a model of atherosclerosis) increased atherosclerotic lesion size and increased adventitial infiltrate of activated lymphocytes (Ostos et al., 2002). This finding suggests that LPS derived from invading pathogens may accelerate or trigger atherosclerosis. Gram-positive bacteria do not posses LPS. however, these bacterial populations posses several alternative PAMPs including peptidoglycans, lipopeptides, lipoteichoic acid, unmethylated DNA fragments (specifically, oligomer containing CpG dinoucleotides in the correct sequence context) and double-stranded RNA (Karlsson et al., 2002). Although somewhat weaker than LPS in their immune potency, each of these molecules elicits an innate immune response (Muhlradt et al., 1997, Weidemann et al., 1997, Morath et al., 2001, Wagner, 2002).

Viruses possess four classes of PAMPs including double-stranded RNA (dsRNA), single strandad RNA (ssRNA), unmethylated CpG DNA and envelope glycoproteins. There are many common elements in the cellular responses to Gram-negative, Gram-positive and viral PAMPs, including the recruitment of leukocytes and the release of chemokines and cytokines, such as TNFα. However, our understanding of how PAMPs and the resulting immune activation affect CVD and RA pathophysiology remains largely limited.

1.2.2 Pattern recognition receptors

Initiation of an effective immune response is reliant upon first recognising the pathogenic article. The initial sensing of infection is mediated by germline-encoded pattern recognition receptors (PRRs) of the innate immune system (Takeuchi and Akira, 2010).

Several classes of PRRs exist, including Toll-like receptors (TLRs), NOD-like receptors, RIG-I-like receptors and C-type lectin receptors. This limited repertoire of PRRs is in contrast to the huge repertoire of rearranged receptors utilised by the acquired immune system. The innate immune system is the principal contributor to the acute immune reaction triggered by infection (Akira et al., 2006). Intracellular signalling cascades

triggered by PRRs leads to the expression of immune and inflammatory mediators that coordinate the effective removal of pathogens.

PRRs share several common characteristics: (1) PRRs recognise microbial components known as PAMPs that are essential for survival and replication of the microbe; (2) PRRs are constitutively expressed in the host and detect microbes regardless of their lifecycle; (3) PRRs are germline encoded, nonclonal and posses no immunologic memory (Akira et al., 2006).

The remainder of this section will briefly review the mechanisms by which TLRs and NOD-like-receptors (NLRs) detect the presence of microbial insults, and how the resulting PRR signals stimulate an immune response. A brief outline of which PAMPs the principal PRRs are capable of detecting is provided in *table 1.1*.

The TLR family is evolutionarily conserved and is responsible for sensing microbial components outside of the cell and in intracellular lysosomes and endosomes (Chaturvedi and Pierce, 2009). Twelve members of the TLR family have been identified in humans. TLRs are characterised by extracellular domains containing leucine rich repeat (LRR) motifs and a transmembrane signalling domain termed the Toll/IL-1R homology (TIR) domain (Bowie and O'Neill, 2000). LRR is composed of 20 to 30 amino acid stretches that are rich in the hydrophobic amino acid leucine. Typically each repeat unit is composed of a β-strand α-helix structure and the assembled domain, composed of many repeats, has a horseshoe like conformation (Kobe and Deisenhofer, 1994). It has been widely considered that ligands would bind to the concave structure of the horseshoe structure (Kobe and Kajava, 2001). However, following characterisation of the three-dimensional structure of the human TLR3 LRR motif it has been suggested that negatively charged dsRNA (a PAMP recognised by TLR3) is likely to bind to the outer convex surface of the horseshoe-like conformation (Choe et al., 2005). It is currently unknown if this observation can be applied to the other members of the TLR family.

The TLR family can be divided into subfamilies based on their primary sequences. Each subfamily detects related PAMPs. The highly related subfamily consisting of TLR7, TLR8 and TLR9 recognises nucleic acids, whereas the subfamily consisting of TLR1, TLR2 and TLR6 recognises lipids (*table 1.1*).

The signalling pathway used by the TLR family is highly homologous to that used by the IL-1R (IL-1 receptor) family (Akira and Takeda, 2004). TLRs and IL-1R interact with

MyD88 (myeloid differentiation factor 88) through the TIR (Toll/IL-1R) domain. Following TLR stimulation, MyD88 binds the cytoplasmic TIR domain, where it facilitates the association of TRAF6 (TNF receptor-associated factor 6) and IRAKs (IL-1R associated kinases) (Vallejo, 2011). TRAF6 association activates TAKs (transforming growth factor *β*-activated kinases), which initiates a kinase cascade resulting in the phosphorylation of inhibitory protein I*k*B (Hacker and Karin, 2006). Phosphorylated I*k*B dissociates from the complex and is rapidly targeted for degradation, resulting in the activation of the transcription factor NF-*k*B, which translocates to the nucleus and initiates the expression of several inflammatory cytokines. Although MyD88 has been suggested to be involved in all TLR signalling, TLR3 appears to transduce signals mainly using a MyD88-independent pathway, as the activation of NF-*k*B by dsRNA can occur in the absence of MyD88 (Alexopoulou et al., 2001). Furthermore, subtle differences to the signalling pathway of each TLR exist, thus supporting slightly different gene expression patterns for each TLR.

As well as PAMPs, the PRRs are able to recognise certain endogenous self molecules. For example, β-defensins (Biragyn et al., 2002), heatshock proteins (Ohashi et al., 2000) and a number of extracellular matrix proteins (Okamura et al., 2001) have been reported to activate TLR4 and stimulate inflammation. It has been speculated that these endogenous molecules act as danger signals released from stressed or dead cells to trigger a pro-inflammatory response (Matzinger, 2002).

In addition to being expressed by immune cells, TLRs are also expressed by tissues of the cardiovascular system. There is accumulating evidence that TLRs, notably TLR2 and TLR4, are involved in atherosclerotic development and progression (Lin et al., 2009). TLR2 was shown to be over-expressed in arterial endothelial cells in atherosclerosis prone $LDLr^{-/-}$ mice, and diet-induced hyperlipidaemia further increased this expression (Mullick et al., 2008). TLR4-expressing macrophages have been identified in human atherosclerotic lesions and at sites of plaque rupture (Ishikawa et al., 2008). However, the specific role of individual TLRs in initiating and maintaining pathological responses in the cardiovascular system remains to be defined.

While TLRs constitute the main sensors for detection of extracellular microbes, recent findings suggest that NLRs act as intracellular surveillance molecules (Meylan et al., 2006, Fritz et al., 2006). Several proteins of the highly conserved NLR family have been demonstrated to act as PRRs for the initiation of innate immune responses upon pattern specific microbial sensing (Inohara and Nunez, 2003). Two members of the NOD family

(NOD1 and NOD2) recognise specific bacterial components D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP) respectively (*table 1.1*). NOD proteins are members of a family that includes the apoptosis regulator APAF1 (apoptotic protease activating factor 1), mammalian NOD-LRR proteins and plant-disease-resistance gene products. Similar to TLRs, several NOD proteins have been implicated in the induction of NF-*k*B. In addition, a role for NOD proteins in regulation of caspase activation and apoptosis has been indicated (Warren et al., 2008). Interestingly, genetic variation in the genes encoding NOD proteins in humans is associated with inflammatory disease and increases susceptibility to bacterial infection (Hampe et al., 2001, Hoffman et al., 2001).

PRR	**Localisation**	**Ligand**	**Origin of ligand**
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, virus, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, virus, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, virus
TLR8	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa
NLR			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria

Table 1.1 PRRs and their ligands

1.2.3 Inflammation

As will become apparent in later sections, RA is a prototypical inflammatory disease (*section 1.3.1*). Furthermore, understanding of atherosclerosis has evolved from a passive process resulting in narrowing of the lumen to a dynamic inflammatory process (Chung et al., 2007) (*section 1.4.4*). Inflammation plays a pivotal role in an orchestrated immune response and can be activated by several mechanisms. But what exactly is inflammation, and how does it function?

The inflammatory process is the characteristic response of tissue to infection or cellular injury. Inflammation functions to destroy, dilute or isolate the damaging agents and limit damage to the tissue. Furthermore, inflammation is necessary for the mediation of immune responses and the maintenance of homeostasis. At the clinical level, the inflammatory response is usually accompanied by tenderness, oedema, erythema and pain. These associated symptoms occur due to increased blood flow to the site of inflammation, recruitment of activated leukocytes and fluid leakage into surrounding tissue. The actions of inflammatory mediators and cytotoxic lymphocytes function to remove damaged tissue and pathogens, supporting healing, restoration of function and homeostasis.

Inflammation is characterised by (1) vasodilation of local blood vessels with subsequent excess blood flow; (2) accumulation of fluid in interstitial space due to increased permeability of surrounding capillaries; (3) migration of monocytes and granulocytes into the affected tissue; (4) swelling of the cells within the tissue. Some of the key pro-inflammatory molecules include serotonin, histamine, bradykinin, blood clotting factors, the complement cascade and lymphokines secreted by sensitised cells.

1.2.4 The acquired immune system

The innate immune system is not an isolated response. Instead, several components of the innate immune system cooperate with the acquired immune system, resulting in a highly integrated immune response.

The acquired immune response involves the proliferation of antigen-specific B and T cells. Activation of these cells is dependent upon antigen-presenting cells of the innate immune system effectively presenting class I and class II MHC antigens. This is predominantly

performed through the action of macrophages and dendritic cells. Class I and class II MHC presenting antigen will activate cytotoxic $CD8^+$ and $CD4^+$ helper T cells respectively (Harding et al., 2003).

MHC class I molecules are expressed on all nucleated cells. The presentation of foreign antigen and MHC molecule allows infected cells to establish intercellular contacts with cytotoxic $CD8^+$ T cells. When exposed to such cells effector $CD8^+$ T cells release cytotoxins that form pores in the target cell membrane, resulting in lysis.

$CD4^+$ T cells, or helper T cells, are immune-response mediators. Unlike $CD8^+$ T cells these cells have no phagocytic or cytotoxic capabilities. Upon recognition and binding of class II MHC expressing foreign antigen, $CD4^+$ T cells release specific cytokines which influence the activity of an array of immune cells.

The principle function of B cells is to produce antibodies. Antibodies serve several functions including, preventing microbes adhering to host cells, neutralising toxins, opsonising microbes, activating complement, and sensitising infected cells for antibody-dependent attack by natural killer cells (NK cells). Similar to the innate and acquired immune system, T and B cells interact with each other. Antigen recognised by B cells is internalised and processed for MHC class II expression on the B cell surface. This antigen is then presented to a primed specific T cell. The T cell then produces B cell growth factors which stimulate B cell division and maturation, resulting in the formation of antibody-secreting cells. Following an immune response, two types of differentiated B cells persist in a B cell memory pool: plasma cells, which confer immediate protection by the secretion of specific antibodies and memory B cells, which confer a rapid and enhanced response to secondary challenge (Lanzavecchia and Sallusto, 2009).

B cells play several roles in the pathogenesis of RA. They are a source of rheumatoid factors and anti-citrullinated protein antibodies, which activate complement and contribute to the formation of immune complexes in the joints (Silverman and Carson, 2003). B cells both produce and respond to cytokines and chemokines that promote leukocyte infiltration into the joints. Furthermore, germinal-like structures consisting of T and B cells clustered in aggregates around interdigitating dendritic cells are observed in the synovium of about 60% of RA patients (Silverman and Carson, 2003). Generally speaking, the presence of lymphoid infiltration correlates with clinical disease in RA (Weyand and Goronzy, 2003). Furthermore, evidence suggests that the mechanisms of immune-mediated tissue damage

are shared between the synovium and the atherosclerotic lesion, predisposing RA patients to CVD (Weyand et al., 2001).

1.3. Rheumatoid arthritis

Rheumatoid arthritis (RA) is the most prevalent inflammatory arthritis and is characterised by systemic inflammation, persistent synovitis and autoantibodies leading to severe disability and premature mortality (Scott et al., 2010, Pincus et al., 1984). RA principally affects the peripheral joints, which can lead to cartilage damage, bone erosion, and can subsequently disrupt bone integrity.

In industrialised countries, RA affects 0.5-1% of the adult population and is a major cause of disability. The incidence of RA is three times greater in women than men. Rarely will RA occur in individuals under 15 years of age, but following this age the risk of RA increases until 80 years of age (Majithia and Geraci, 2007). Severity of disease can vary enormously depending on the number of joints affected, the extent of inflammation and the rate of progression.

Despite RA being considered an autoimmune disease the exact initiating stimulus remains unknown (Resch, 2008). However, it is likely that RA results from a complex interaction between environmental and genetic factors. An initial trigger, possibly infectious or autoimmune, initiates joint inflammation, with subsequent interactions between cells of the immune system, their cytokines, growth factors and proteinases leading to joint destruction (Majithia and Geraci, 2007) (*Section 1.3.1*).

There is no single test to confirm the diagnosis of RA. Instead, classification criteria devised by the American College of Rheumatology (ACR) and the European League Against Rheumatology (EULAR) (Aletaha et al., 2010) aim to distinguish individuals with RA from those with other types of established joint disease (*table 1.2*). These criteria replace previous long-standing ACR diagnosis criteria (Arnett et al., 1988). The updated criteria require measurement of the acute phase respondents, namely C-reactive protein (CRP) and the erythrocyte sedimentation rate (ESR). Furthermore, measurement of anti-citrullinated protein antibody (ACPA), an autoantibody against citrulline which can precede physical RA manifestation by several years (Nielen et al., 2004, Rantapaa-Dahlqvist et al., 2003), is required. Together these updates support earlier diagnosis of RA and will undoubtedly result in improved clinical outcomes over the long term.

	Score
A. Joint involvement *	
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5
B. Serology (at least one test result is needed for classification)	
Negative RF and negative ACPA	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
C. Acute-phase reactants (at least one test is needed for classification)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
D. Duration of symptoms	
<6 weeks	0
≥6 weeks	1

Table 1.2 ACR-EULAR classification criteria for rheumatoid arthritis (Aletaha et al., 2010).

The scores from categories A-D are added; a score of ≥6 is needed to diagnose a patient as having definite RA. Patients are recommended to undergo these tests if they have at least one joint with definite clinical synovitis and if the observed synovitis cannot be better explained by an alternative disease.

*'Large joints' refer to knees, hips, ankles, shoulders and elbows. 'Small joints' refers to the proximal interphalangeal joints, metacarpophalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints and wrists.

1.3.1 Pathogenesis of rheumatoid arthritis

Despite intensive research the cause of RA remains unknown. However, understanding of the pathogenesis of RA has advanced significantly in recent years. RA is best regarded as a clinical syndrome involving several different inflammatory pathways that ultimately converge, resulting in persistent synovial inflammation and associated bone and cartilage damage (Scott et al., 2010, van der Helm-van Mil and Huizinga, 2008).

1.3.1.1 Inflammation in RA

Inflammation is typically a tightly regulated process involving both pro-inflammatory mediators and anti-inflammatory mediators. In RA an imbalance exists between these two mediator systems, leading to cellular damage and tissue destruction (Choy and Panayi, 2001).

The synovial membrane in patients with RA is characterised by an infiltrate of inflammatory cells, principally CD4+ T cells, which orchestrate cell-mediated immune responses. Antigen-activated CD4+ T cells stimulate monocytes, macrophages and synovial-like fibroblasts to produce pro-inflammatory cytokines such as IL-1, IL-6 and TNFα (Aggarwal, 2003).

The overproduction of tumour necrosis factor (TNFα) has been identified as a key contributor to the inflammatory and tissue-destructive pathways in RA (Feldmann and Maini, 2003). TNFα is overexpressed in the synovial fluid as well as the synovial membrane of RA patients (Digiovine et al., 1988). Furthermore, expression of the TNFα receptor is also up-regulated in the synovial membrane of RA patients and appears to co-localise with areas of bone erosion (Alsalameh et al., 1999). Further evidence to support the direct role of TNFα in RA comes from transgenic mice, where overexpression of TNFα alone resulted in inflammation and rheumatoid-like lesions in the joints (Keffer et al., 1991). Importantly, inhibiting the biological activity of TNFα has revolutionised the treatment of RA and substantially improved clinical outcomes.

Furthermore, TNFα and IL-1 stimulate mesenchymal cells (synovial fibroblasts, chondrocytes and osteoclasts) to release tissue degrading matrix metalloproteinases (Shingu et al., 1993).

Whereas IL-1, IL-6 and TNFα initiate and maintain inflammation, other cytokines such as IL-10 and IL-4 dampen it (vanRoon et al., 1996). Both IL-10 and IL-4 attenuate the production of TNFα and IL-1 and have been shown to inhibit cartilage damage (Vanroon et al., 1995, Sugiyama et al., 1995). Although both IL-10 and IL-4 have been found in the synovial fluid of RA patients, the amount is thought to be insufficient to protect from inflammation (Katsikis et al., 1994, Taki et al., 2000).

1.3.1.2 Autoantibodies in RA

Rheumatoid factor (RF) is the classic autoantibody in RA. IgM and IgA rheumatoid factors are key pathogenic markers directed against the Fc fragment of immunoglobulin (IgG). RF titre has been demonstrated to correlate with synovitis, joint damage and disability (Shmerling and Delbanco, 1991). However, experimental studies have so far failed to attribute a pathogenic role to RF in RA. Additional types of antibodies are those directed against citrullinated peptides (ACPA). Although most ACPA-positive patients are also positive for RF, ACPA seems more specific and sensitive for diagnosis than RF and provides superior predictive information on joint destruction and disease activity (van der Linden et al., 2009, Fathi et al., 2008). Autoantibodies are discussed further in *section 1.5.4*.

1.3.1.3 Genetics

Although not a feature of the research carried out for this thesis, it must be highlighted that there is extensive evidence for a genetic component in the aetiology of RA. A study of 91 monozygotic (MZ) and 112 dizygotic (DZ) twins revealed that 15.4% of the MZ and 3.6% of the DZ pairs were disease concordant (Silman et al., 1993). Furthermore, familial clustering tests demonstrated that there is an increased risk of RA in first- and second-degree relatives of patients (Grant et al., 2001). Owing to such studies, genetic factors are estimated to account for ~60% of the variation in liability to disease (MacGregor et al., 2000).

The HLA-DRB1 gene, which contributes to the major histocompatability complex (MHC), is the best understood genetic risk marker in RA (Williams et al., 1995). Indeed, genome-wide searches using micro-satellite loci have confirmed a linkage between RA and the

HLA-DR allele polymorphisms (Cornelis et al., 1998, Delgado-Vega and Anaya, 2007). Several non-HLA loci have also been demonstrated to exhibit genetic linkage to RA, including the 18q21 region of the TNFRSF11A gene, which encodes the receptor activator of nuclear factor kappaB (RANK), a membrane protein involved in bone resorption in RA (Jawaheer et al., 2003).

1.3.2 Co-morbidities in RA

The pathophysiology of RA is fairly well characterised, but less understood are the co-morbid conditions that are associated with RA. Physicians and researchers are interested in RA co-morbidity because of the potential effect on the quality of life (QOL) and the RA prognosis and outcome (Michaud and Wolfe, 2007). It is customary to think of the functional status of RA patients as being directly related to RA pathophysiology; however, functional status is also strongly influenced by co-morbid conditions.

RA patients have, on average, a 10 to 15 year decreased life expectancy compared to the general population (Pincus and Callahan, 1986, Wolfe et al., 1994). CVD is the leading cause of mortality in RA, with over half of all reported mortalities in RA cohorts being explained by CVD events (Goodson, 2002). Enhancing our understanding of why CVD burden is elevated in RA is fundamental to improve clinical outcomes and forms the basis of the research outlined in this thesis. Atherogenesis in rheumatology is discussed in greater detail in *section 1.5*.

In cohorts of RA patients, infection is considered the second greatest contributor to mortality (Franklin et al., 2007, Goodson et al., 2002) and the rate of infection-related mortality is typically 4 to 6 times greater than that of the general population (Sihvonen, 2006, Au et al., 2011). This association has implications for the research described in *chapter 4*, which characterises bacterial species present in the aortic adventitia of patients with RA.

Although not the subject of the research presented in this thesis, epidemiological studies have also demonstrated that RA is associated with pathologies such as osteoporosis, gastrointestinal disease, cataracts, psoriasis and certain cancers. Despite mortality rates resulting from these associated conditions being slight in comparison to CVD, future research into these co-morbidities could lead to development of therapeutics that significantly increase the quality of life experienced by RA patients.

Direct evidence to support CVD as a major pathology in RA comes from stress myocardial perfusion imaging. This relatively simple technique allows clinicians to quantify blood flow to the myocardium. These studies demonstrated that 50% of RA patients aged 40 to 70 years exhibited definitive signs of cardiac ischemia (principally caused by atherosclerosis), whereas only 27% of controls matched for age, sex and all classical cardiovascular risk factors exhibited similar signs of cardiac ischemia (Banks et al., 1998). This clinical observation occurred roughly 10 years earlier in the RA cohort indicating that RA itself (or the pathogenic mechanism) is an independent risk factor for CVD, attributable to accelerated atherosclerosis.

Inflammation is known to play a role in the pathophysiology of atherosclerosis (Ross, 1999, Alexander, 1994, Pasceri and Yeh, 1999). In animal models engineered to over-express inflammatory mediators, atherosclerosis severity is increased (Huber et al., 1999) and inflammatory cells and mediators have been found in human atherosclerotic plaque (Naruko et al., 2002). Furthermore, elevated serum concentration of inflammatory markers such as IL-6 (Ridker et al., 2000) and CRP (Ridker, 2005) can predict atherosclerosis. Consequently, elevated atherosclerosis risk could result from the chronic inflammation that accompanies RA.

In order to conceptualise current thinking on elevated atherosclerosis risk in RA, and to introduce new hypotheses, it is important first to understand the pathogenesis of atherosclerosis. The following section (*section 1.4*) will provide *inter alia* an overview of CVD before describing the physiology of atherosclerosis in more detail.

1.4 Cardiovascular disease

1.4.1 The global burden of cardiovascular disease

Cardiovascular disease (CVD) is a broad-spectrum disease that most commonly refers to atherosclerosis, but also includes conditions such as angina and stroke. CVD results from an accumulation of atherosclerotic plaques within the walls of the arteries that supply the myocardium. In 2008 the World Health Organization (WHO) ranked CVD as the premier cause of global death, accounting for 12.2% of all recorded deaths (WHO, 2011). This

trend is expected to continue indefinitely (Murray and Lopez, 1997a, Murray and Lopez, 1997b). Every 25 seconds an American citizen will experience a coronary event and every 60 seconds someone will die as a result of such an event (Roger et al., 2011).

CVD is causally linked to lifestyle. Poor diet, lack of exercise and smoking have been popularised in the media and established in the literature to be major cardiovascular risk factors (Karasek et al., 2010, Gordon et al., 1974, Wildman et al., 2011). The last few decades of urbanisation has led to changes in behavioural patterns towards activities that increase CVD burden. Reduction in manual labour and greater reliance on motorised transportation coupled with the global uptake of an unhealthy western diet (low fruit and vegetable, high salt and saturated fat) have led to a pandemic of obesity in the developed nations. The WHO estimates that in 2006 66.7% of US adults were overweight (BMI $\geq$ 25 kg/m^2) and that 33.3% were obese (BMI $\geq$ 30 kg/m^2). Data from the 2009 United States Behavioural Risk Factor Surveillance System (BRFSS) of the Centers for Disease Control and Prevention (CDC) (Sherry et al., 2010) documented a 1.1% increase in US obesity between 2007 and 2009. This has led some researchers to infer that by 2050 a vast proportion (approaching 100%) of American citizens will be overweight (Yanovski and Yanovski, 2011).

There has been an epidemiological shift in the developing world which has seen a decrease in deaths due to communicable disease and malnutrition, coupled with an increased burden of degenerative disease – the major contributor being atherothrombotic CVD. This epidemiological shift can be categorised into general stages. The first stage encompasses the least developed countries (e.g. sub-Saharan Africa) where the predominant circulatory diseases are nutritional related disorders of the cardiac muscle and rheumatic heart disease (Yusuf et al., 2001). Second-stage countries (e.g. China) are experiencing a decrease in communicable disease and improvements in nutrition. In China, for example, hypertension-related diseases such as hypertensive heart disease and haemorrhagic stroke are the major contributors to CVD (Chiu et al., 2010). Countries in the third stage of the epidemiological shift (e.g. Latin America, urban India) are experiencing improved life expectancy. Here, sedentary lifestyles are being increasingly adopted, smoking and high fat diets are increasing, and as a result noncommunicable diseases predominate, with the greatest contributor to mortality being atherosclerotic CVD (Reddy, 1999). For the majority of developing nations the increased prevalence of CVD accompanies the continuing burden from nutritional and infectious disease. This has been aptly termed the "double burden". The fourth stage, applicable to highly developed nations, is in all senses

similar to the third stage, however increased investment in diagnosis and treatment has led to a delay of symptoms until a later age (Yusuf et al., 2001).

Prevention and treatment of CVD is therefore a critical clinical and social issue. However, as well as human costs, CVD has major economic consequences. In the United Kingdom in 2006, CVD alone cost the NHS £14.4 billion, representing £250 per capita. 72% of this sum was accounted for by direct hospital care of CVD sufferers and 20% of the cost was due to medication (*figure 1.1*). Accordingly there has been a recent burst of research focusing on identifying individuals at risk of CVD, educating them on the necessary lifestyle changes required and administering suitable prophylaxis such as aspirins and statins. This approach has undoubtedly led to progression in the field, however more research is necessary to enhance our understanding of the molecular mechanisms that trigger and potentiate CVD.

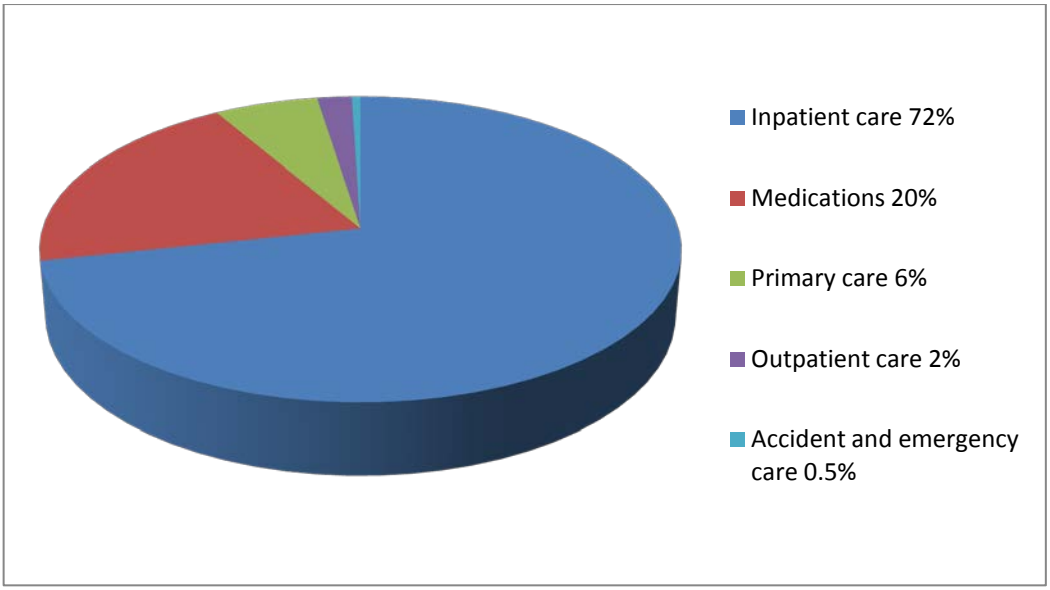


Figure 1.1 Breakdown of NHS spending for diagnosis and treatment of CVD in 2006. *Figure generated using data from the British Heart Foundation Statistics Database.*

1.4.2 Blood vessel structure

In order to understand the pathogenesis of atherosclerosis, it is important to be familiar with the basic structure of a healthy blood vessel. All blood vessels, with the exception of the very smallest, are composed of three distinct layers or tunics that surround the central

blood-containing lumen (*figure 1.2*). The intima forms the innermost layer, which is composed of a single layer of endothelial cells arranged on a thin membrane of laminin, type IV collagen and proteoglycans. An internal elastic lamina separates the tunica intima from the tunica media. The media forms the middle layer of the vessel and comprises vascular smooth muscle cells with a basement membrane of elastic tissue. The tunica adventitia forms the outermost layer of the vessel. The adventitia is principally composed of loose connective tissue comprised of fat cells and a few smooth muscle cells. Nerves and capillaries (*vasa vasorum*), which provide the vessel wall with nutrients, are also present within the adventitia.

It is widely accepted that atherosclerosis is initiated at the endothelial lining of the tunica intima (Kharbanda and MacAllister, 2005, Ross, 1999). The following sections (*section 1.4.3 – section 1.4.3.2*) will describe current understanding of endothelial cells and how these may contribute to atherosclerosis. However, as will become apparent later (*section 1.4.4.2* - the outside in phenomenon) there is a growing body of evidence to suggest that atherosclerosis may originate in the adventitia.

1.4.3 Vascular endothelium

The vascular endothelium is a single-celled layer that lines the interior surface of all blood vessels and forms the interface between the vessel wall and circulating blood in the lumen (*figure 1.2*) (Michiels, 2003). For many years the endothelium was considered a relatively inert container for blood. However, the last two decades have seen research reveal a wealth of endothelial functionality.

Under normal physiological conditions the vascular endothelium allows nutrient exchange but limits the passage of blood and protein. The endothelium also plays vital roles in the maintenance of vascular tone by releasing vasoactive substances such as nitric oxide (NO) as well as contributing to angiogenesis, humoral coagulation and inflammation (Stenvinkel, 2001).

Vascular endothelial cells have threefold functionality in the interaction with leukocytes: (1) they dictate the passage of leukocytes to inflammatory foci; (2) they modulate leukocyte activation; (3) they possess receptors that recognise leukocyte derived molecules, which can result in endothelial cell activation or death (Biedermann, 2001).

Upon recognition of damaged cells or pathogens, neutrophils, macrophages and mast cells become activated. These cells then secrete cytokines such as IL-6, IL-1, TNFα and a range of other pro-inflammatory mediators. These molecules stimulate blood vessels at the site of damage to vasodilate, increase vascular permeability and activate endothelial cells to express adhesion molecules, including vascular cell adhesion molecule-1 (VCAM-1) and inter-cellular adhesion molecule-1 (ICAM-1) (Woodfin et al., 2009). Adhesion molecules recognise and bind to circulating lymphocytes, supporting migration from the vascular lumen to the site of infection and/or injury (Yadav et al., 2003).

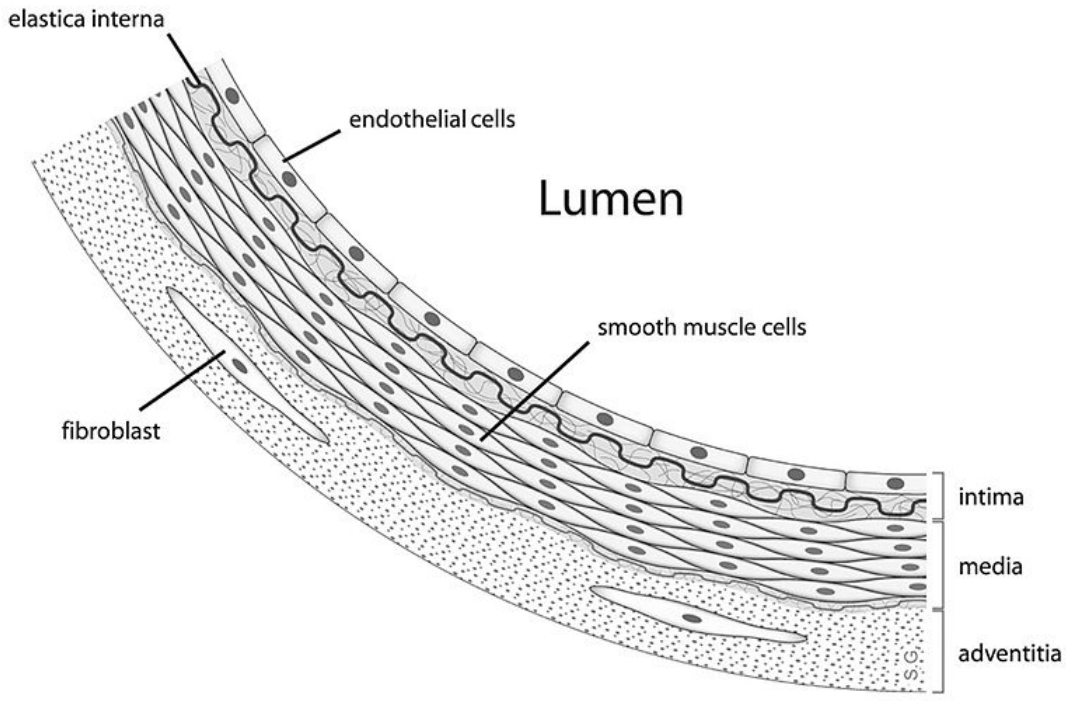


Figure 1.2. Location of endothelial cells in the vessel wall

A single layer of endothelial cells confined to the intimae line the surface of blood vessels, forming an interface between circulating blood and the rest of the vessel. The media makes up the thickest layer of the vessel and is composed principally of smooth muscle cells. The adventitia is composed primarily of connective tissue and also contains nerves and capillaries (vasa vasorum) that serve the vessel wall. Figure adapted from Micheles,2003.

1.4.3.1 Endothelial-dependent leukocyte recruitment

Evidence suggests that the progression of atherosclerosis resembles a chronic inflammatory disease (Hansson, 2005). Several different leukocyte subsets have been identified in the early lesion, including B cells, T cells, monocytes and macrophages, natural killer (NK) cells, dendritic cells (DCs), activated smooth muscle cells (SMCs), mast cells and a range of products secreted from these cells (Charo and Ransohoff, 2006).

A key event in the atherogenic inflammatory response is the endothelial-dependent recruitment of leukocytes to the site of tissue damage and/or infection. The vascular endothelium is responsible for orchestrating the cellular and regulatory mechanisms of leukocyte recruitment to the vessel wall (Rao et al., 2007). Endothelial cell activation by a variety of proinflammatory cytokines (e.g. TNFα, IL-1β, IFNγ), thrombin, haemodynamic factors, bacterial endotoxin and viruses can lead to loss of endothelial function, local thrombosis and rapid leukocyte recruitment (Gimbrone, 1999). These inflammatory mediators lead to small blood vessel dilation, resulting in increased blood flow and a slowed course of circulating leukocytes, thus allowing them opportunity to contact the endothelial lining. If unrestricted, these alterations can lead to vascular inflammation and contribute to atherosclerosis (Meredith et al., 1993) and RA (Foster et al., 2010).

A key stage in leukocyte recruitment is the secure adhesion of leukocytes to the surface of the endothelium, allowing migration into the vessel wall. The specificity of leukocyte recruitment to the vascular wall is most probably dictated by the expression of different combinations of adhesion molecules and locally expressed chemoattractants, cytokines and chemokines. Furthermore, only subsets of leukocytes which posses the ability to disrupt endothelial cell junctions including components such as tight junctions (claudins, occludins) and adherens junctions, will be free to pass across the endothelial lining.

1.4.3.2 Endothelial dysfunction

Endothelial dysfunction is an important early event in atherosclerosis, contributing to plaque formation. Accumulating evidence indicates that endothelial dysfunction can be detected before physical changes to the vascular wall (Davignon and Ganz, 2004). Endothelial dysfunction is characterised by a shift in endothelial homeostasis towards a prothrombotic and proinflammatory state with reduced vasodilation (Endemann and Schiffrin, 2004). Mechanisms that contribute to reduced vasodilation include reduced

production of nitric oxide (NO), diminished generation of hyperpolarising factor and oxidative excess (Chen et al., 2001). Production of chemokines such as macrophage chemoattractant peptide-1 and upregulation of endothelial adhesion molecules supports a prothrombotic and proinflammatory response (Griendling and FitzGerald, 2003, Khan et al., 1996).

The diminished production of NO by the endothelium appears paramount in the pathophysiology of endothelial dysfunction. In addition to its vasodilatory effect, NO produced by the endothelium stimulates neovascularisation and angiogenesis (Aicher et al., 2004), promotes endothelial growth and survival (Kawasaki et al., 2003), limits leukocyte adherence to the endothelium (Gauthier et al., 1995), and restricts platelet aggregation and reduces risk of thrombosis (De Graaf et al., 1992). Interestingly, CRP (an established biomarker for CVD and RA) significantly downregulates endothelial nitric oxide synthase (eNOS) and disrupts eNOS mRNA expression, resulting in reduced NO and increased inflammatory cell adhesion (Verma et al., 2002, Venugopal et al., 2002).

1.4.4 Formation of the atherosclerotic lesion

Atherosclerosis is a chronic inflammatory disorder resulting from interaction between modified lipoproteins, monocytes and T cells as well as resident cells of the vessel wall (Glass and Witztum, 2001). Chronic inflammation ultimately leads to development of the atheromatous lesion. However, the site of atherosclerosis initiation has been the subject of debate in recent years. The following two sections (*Sections 1.4.4.1 and 1.4.4.2*) will summarise the two conflicting hypotheses. This is followed by an overview of the macroscopic stages of atherosclerosis progression, which is applicable to both models of disease initiation.

1.4.4.1 From intima to adventitia

The theory that atherosclerosis is initiated at the intima is the most widely accepted mechanism to explain atherosclerosis pathogenesis. Owing to the direction of pathogenesis, this theory is commonly referred to as ‘inside-out’. Accumulation of LDL in the subendothelial matrix has been described as a primary initiator of atherosclerosis (Steinbrecher et al., 1990). Elevated lipid accumulation within large arteries allows low-

density lipoprotein (LDL) to diffuse freely through endothelial cell junctions. Retention of LDL at the site of atherosclerosis involves interaction between apolipoprotein B (ApoB) and matrix proteoglycans within the intima of the vessel wall. While resident in the tunica intima, LDL will undergo modification by oxygen radicals and enzymes, forming oxidised LDL (oxLDL). Upon exposure to oxLDL, endothelial cells express a variety of surface molecules, including intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1) and P-selectin, which recruit leukocytes into the intima (Hansson and Libby, 2006).

Monocytes recruited to the activated endothelium are exposed to arterial M-CSF (Qiao et al., 1997) and differentiate into macrophages. Macrophages will bind oxLDL through an array of scavenger receptors, principally CD36, SRA-1, SRB-1 and MARCO (Collot-Teixeira et al., 2007, Naito et al., 1992, Sakaguchi et al., 1998). Lipid particles are then taken up via a range of endocytic pathways (Kruth, 2001) to form foam cells. Innate immune recognition of oxLDL epitopes prompts cytokine-mediated recruitment of lymphocytes that subsequently promote an adaptive immune response responsible for the chronic inflammation observed in atherosclerosis (Keaney, 2011). The establishment of persistent arterial inflammation underpins the progression of atherosclerosis, resulting in the formation of a lipid-rich necrotic core and a fibrous cap. These later stages in atherosclerosis development are discussed in more detail in *Section 1.4.4.3*.

1.4.4.2 From adventitia to intima

A growing body of research supports a novel ‘outside-in’ hypothesis (first proposed by Maiellaro & Taylor, 2007) in which vascular inflammation is initiated in the tunica adventitia and progresses inwards to the luminal surface (Maiellaro and Taylor, 2007). During CVD pathogenesis different immune cells, including monocytes, macrophages and T cells, are resident in the adventitia (Wick et al., 1997, Capers et al., 1997). The accumulation of these cell populations in the adventitia results in the local expression of growth factors, chemokines and inflammatory cytokines similar to that observed in the inside-out mechanism (Jabs et al., 2007). According to the ‘outside-in’ hypothesis (*figure 1.3*) expression of inflammatory mediators signal adventitial fibroblasts, which are responsible for the synthesis of extracellular matrix and collagen, to undergo a phenotypic switch into migratory myofibroblasts. The myofibroblasts transform into a contractile myofibroblast which secrete chemokines such as ccl-2, to recruit further monocytes, before

finally undergoing apoptosis in the media. The fibroblasts that remain in the adventitia will secrete a range of cytokines and chemokines, resulting in pronounced leukocyte infiltrate. Inflammatory infiltration into the adventitia has been suggested to occur through the *vasa vasorum*. Upon exposure to inflammatory mediators the *vasa vasorum* will upregulate expression of adhesion molecules such as VCAM-1 and P-selectin.

Following pronounced inflammatory infiltration, germinal centres are formed. Antigen presenting cells interact with these lymphoid organ-like clusters of lymphocytes supporting the generation of antibodies against inflammatory antigens. Antigen presenting cells also interact with T cells, transforming them into pro-inflammatory Th1 cells. The cytokines and chemokines secreted by activated lymphocytes recruit leukocytes from the lumen into the intima and subsequent transformation into macrophages. At this point, both the intima-adventitia and adventitia-intima directional models share the same sequence of events. Macrophages uptake oxLDL, forming foam cells. These cells then form apoptotic bodies and further release pro-inflammatory molecules, at which point the early atheromatous lesion becomes visible followed by the fibrous plaque.

It is apparent that this theory provides no clear indication as to why immune cells initially present in the adventitia surmount an aggressive proinflammatory response. This theory does however support a hypothesis where by autoantigens and/or microbial pathogens may reside in the adventitia and trigger a localised inflammatory response. This concept will be reviewed further in *sections 1.5.4 and 1.5.5* and is the subject of some of the research outlined in this thesis (*chapters 3 and 4*).

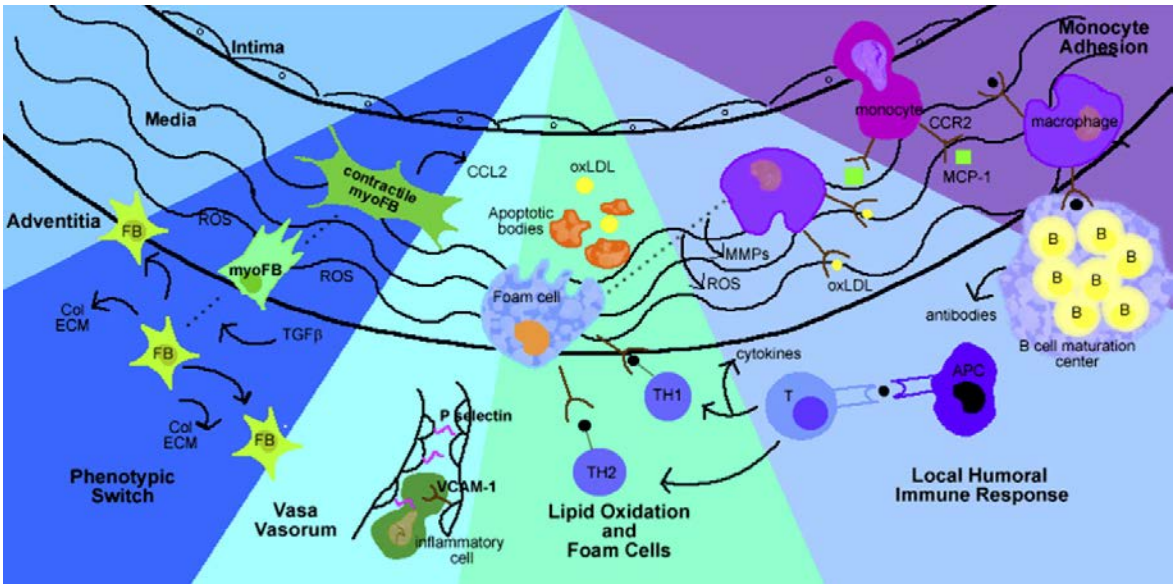


Figure 1.3 Atherosclerosis initiated in the adventitia

The expression of inflammatory molecules leads to a phenotypic switch in fibroblasts to migratory myofibroblasts. Leukocyte recruitment to the adventitia supports a humoral response and allows further influx of leukocytes from the endothelium. Macrophages present in the intima and media then uptake oxLDL and form foam cells, resulting in the early atherosclerotic lesion. Figure from (Maiellaro and Taylor, 2007)

1.4.4.3 Clinical manifestations of atherosclerosis

The previous two sections outlined two alternative hypotheses of the mechanism behind atherosclerosis initiation. At the point of initiation, atherosclerosis remains asymptomatic. It is only after several years that chronic inflammation of the vessel wall results in clinical manifestations (Ross, 1993).

During atherosclerosis progression the structure of the vessel wall is altered. SMCs from the media migrate to the intima. The proliferation of both migratory and resident intimal SMCs coupled to the synthesis of extracellular matrix molecules such as elastin, collagen and proteoglycans forms the foundation of the atherosclerotic plaque (Libby et al., 2011). Foam cells and SMCs undergo apoptosis in advancing lesions releasing lipid into the extracellular matrix. The accumulation of apoptotic foam cells and SMCs under the intima forms a plaque visible as a fibrous cap covering the surface of the lesion. Extracellular lipid accumulates at the central region of the plaque to form a necrotic core, which, if large enough, can occlude blood flow through the vessel (*figure 1.4*).

Owing to the structure of the plaque and the haemodynamic stress experienced during systolic expansion and diastolic contraction, the fibrous plaque is prone to rupture. Plaque rupture exposes tissue factors in the plaque interior to blood coagulation factors in the lumen resulting in thrombus formation (Didangelos et al., 2009). Thrombi can extend into the lumen and are capable of completely occluding arteries resulting in myocardial infarction. Alternatively, thrombi can detach and occlude smaller downstream branches of the circulatory system, causing thromboembolism (i.e. stroke).

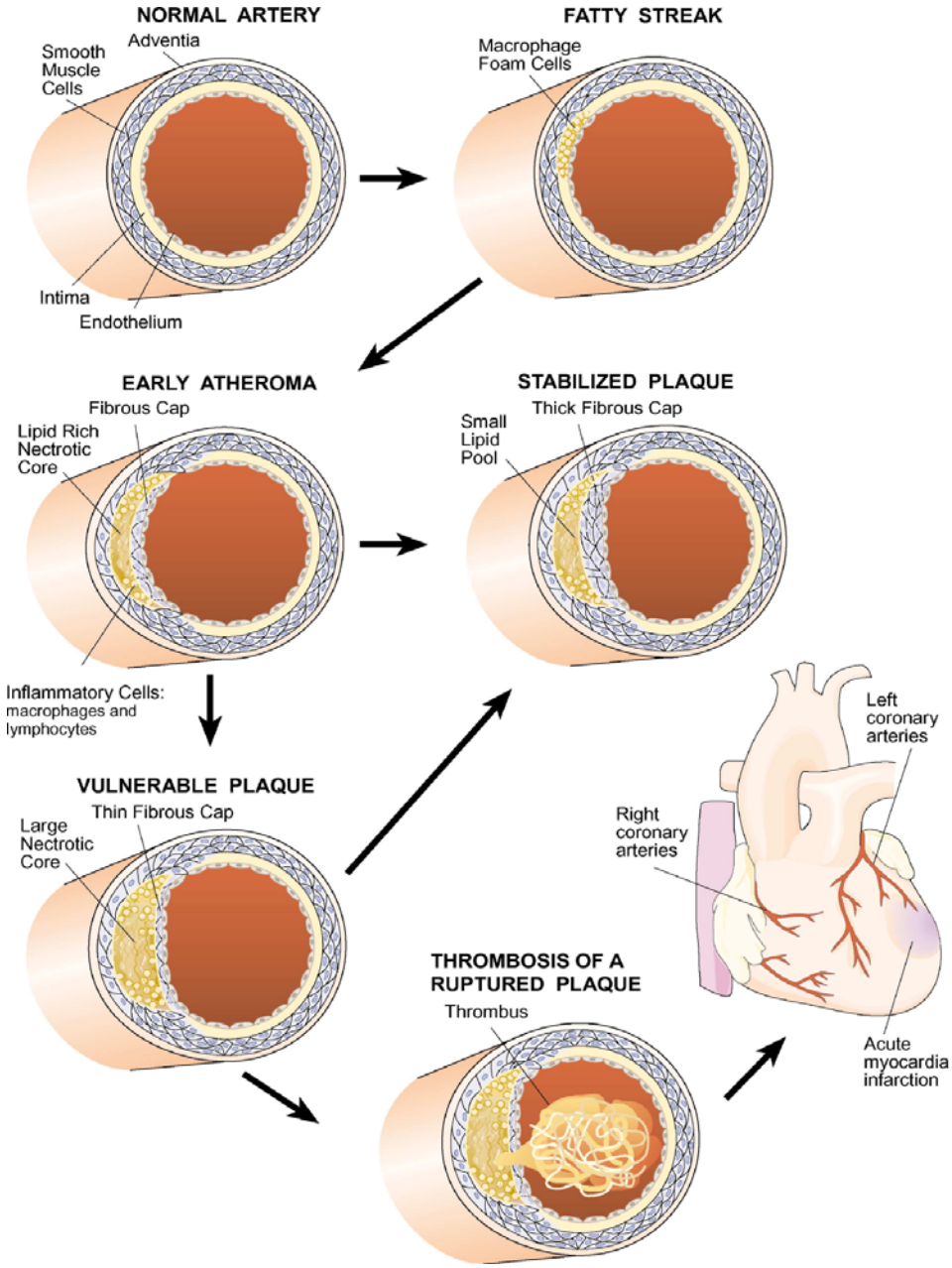


Figure 1.4 The stages of atherosclerosis

The earliest lesion consists of subendothelial accumulation of lipid-laden macrophages (foam cells). Such lesions are not clinically significant, but form the precursor for a more advanced lesion characterised by the accumulations of SMCs and necrotic debris. The necrotic core of advanced lesions is typically enclosed by a fibrous cap consisting of extracellular matrix and SMCs. Advanced lesions can grow sufficiently large to block blood vessels. More commonly, advanced lesions become unstable resulting in rupture followed by thrombus. Such clinical complication can result in myocardial infarction or stroke. Figure adapted from (Lusis et al., 2004).

1.5 Atherogenesis in RA

1.5.1 Exploring the link

It is widely documented that RA patients die prematurely (Wolfe et al., 1994, WallbergJonsson et al., 1997). The gap in mortality between RA patients and the general population is widening because RA patients have not experienced the improvements in survival evident in the general population over the past four decades (Gonzalez et al., 2007). A recent meta-analysis of observational studies indicates that increased CVD burden is the principle contributor to elevated mortality rates in RA patients (Avina-Zubieta et al., 2008). Importantly, despite the obvious clinical importance of CVD in RA, understanding why these diseases are associated remains elusive.

The following sections will briefly review the potential contribution of traditional and non-traditional risk factors to the excess CVD burden observed in RA. Particular attention has been paid to areas that are relevant to the research outlined in this thesis. As well as exploring current popular thinking, several novel hypotheses are suggested.

1.5.2 Traditional risk factors shared between RA and CVD patients

CVD risk factors provide strong predictors of cardiovascular outcomes that contribute to CVD in the general population (Kannel and Schatzkin, 1985). The prevalence of traditional CVD risk factors in RA patients compared to controls has been well described and their relative contribution to excess CVD in RA is continuously being assessed (Solomon et al., 2004, Semb et al., 2010). However, epidemiological work often shows unexpected or paradoxical results and associations, some of which may be explained by biological phenomena peculiar to the RA population (Gonzalez et al., 2008).

The most striking of these unexpected epidemiological observations comes when considering body mass index (BMI). BMI is a well established risk factor for CVD and in the general population mortality as a result of CVD reaches its highest among people in the top BMI categories ($>30 kg/m^2$) (Fontaine et al., 2003). However, within RA patients an inverse association between BMI and mortality has been observed (Escalante et al., 2005) with low BMI resulting in a threefold increased risk of CVD mortality (Kremers et al., 2004).

Emerging information suggests a relationship between obesity and systemic inflammation (Lehrke and Lazar, 2004). High BMI principally results from increases in both the number and size of adipocytes. Adipose tissue was previously considered to act as a simple fuel store, but is now viewed as a complex endocrine system capable of secreting pro-inflammatory and pro-atherosclerotic cytokines such as IL-6 and TNFα (Ahima and Flier, 2000). Macrophages have also been demonstrated to infiltrate adipose tissue in states of obesity (Weisberg et al., 2003), further perpetuating a pro-inflammatory state. This apparent immunogenicity within adipose tissue may play a role in the 'outside-in' theory of atherosclerosis progression (*section 1.4.4.2*), as the majority of adipocytes reside in the adventia, the outer most layer of the vessel wall. Although these observations may help to explain why elevated BMI increases the risk of CVD in the general population, the protective effect of adiposity in RA patients remains paradoxical. In order to abet understanding of why this anomaly occurs it would be useful to correlate adventitial inflammation with BMI in both RA patients and the general population.

The relative contribution of other traditional CVD risk factors, including smoking, hypertension, hyperlipidaemia, family history of CVD, diabetes mellitus and the male gender appears to be less in RA patients compared to the general population (Gabriel, 2010) (*figure 1.5*).

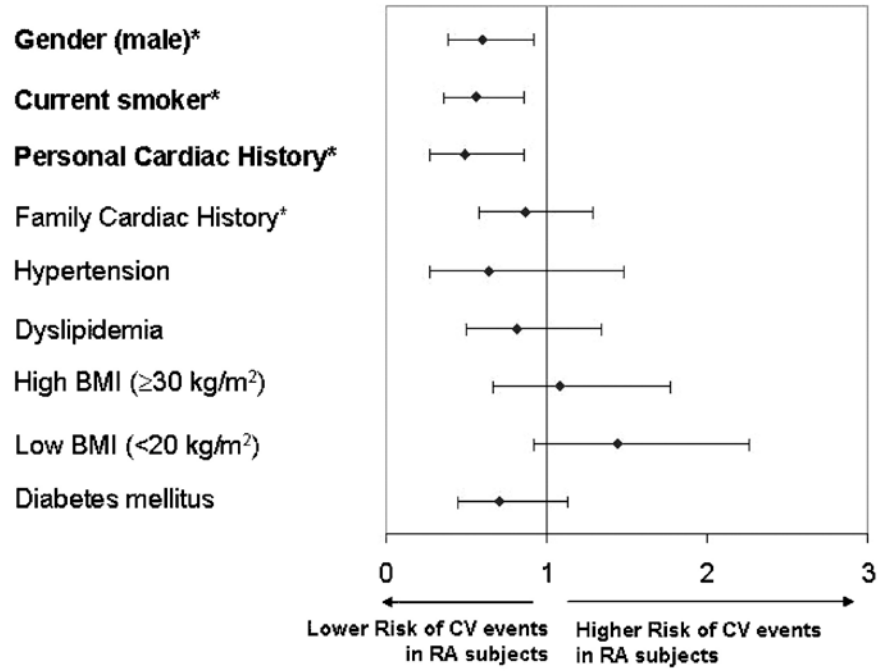


Figure 1.5 Impact of traditional risk factors on endpoint CVD in RA compared to the general population

Figure reproduced from Gabriel (2010); original data from Gonzalez et al (2008).

Undoubtedly, the anomaly in CVD risk factors between RA patients and the general population would benefit from further research in order to enhance understanding of the mechanisms underlying these relationships. Kitas and Gabriel (2010) postulated that the chronic high-grade systemic inflammation may impart significant CVD risk in RA but not non-RA subjects. They further suggested that such high-grade inflammation could have a dilution effect, resulting in the relative contribution of traditional risk factors appearing small in RA patients (Kitas and Gabriel, 2010).

1.5.3 Systemic inflammation

Accumulating evidence suggests that atherosclerosis is an inflammatory condition with a pathophysiology similar to that observed in RA (Ross, 1999, Pasceri and Yeh, 1999). It has been proposed that RA-driven systemic inflammation could predispose an individual to atherosclerosis (Sattar et al., 2003). During RA pathogenesis, the primary site of inflammation in is the synovial tissue, from which cytokines are released into the systemic

circulation. CVD burden in RA has been found to correlate with the number of inflamed joints (Pincus et al., 1984).

The concept of inflammatory-driven atherogenesis is supported by the composition of the atherosclerotic lesion, which exhibits an abundance of immune cells and inflammatory markers. Pro-inflammatory cytokines have also been shown to play a predominant role in both RA and CVD (Park and Pillinger, 2007). However the levels of pro-inflammatory cytokines detected in CVD patients are significantly lower than those observed in the serum of RA patients (Sattar et al., 2003). Several other features are common in the two diseases, including T cell activation (increased Th1:Th2 cell ratio), elevated serum concentration of acute phase reactants (including CRP and fibrinogen) and the local expression of leukocyte adhesion molecules and endothelin (Snow and Mikuls, 2005).

Endothelial dysfunction is considered to be one of the earliest events in CVD pathogenesis (*section 1.4.3.2*) and leads to increased inflammatory cell adhesion, reduced dilator function and increased coagulation activity (Davignon and Ganz, 2004). Pro-inflammatory cytokines have been shown to impair endothelial function both in dissected human veins and in animal models (Clapp et al., 2004, Aicher et al., 2004).

If systemic inflammation were a causative agent in CVD pathogenesis, it would seem likely that the use of anti-inflammatory RA therapeutics would decrease CVD burden. In a small study involving 25 RA patients, disease-modifying anti-rheumatic drugs (DMARDs) were found to significantly improve coronary microcirculation (Turiel et al., 2010). Similarly, treatment of RA with infliximab, a monoclonal antibody targeting TNFα, has been shown to improve endothelial function in RA patients (Gonzalez-Juanatey et al., 2008, Hurlimann et al., 2002).

These observational studies support the hypothesis that elevation of pro-inflammatory cytokines promotes atherosclerosis; however, they do not explain how cytokines that are elevated as a result of RA promote atherogenesis at a molecular level. One suggestion, by Sattar *et al* (2003), is that the mechanism may involve the pleiotropic functions of cytokines, since they mediate numerous metabolic effects in addition to their role in regulating immune responses and inflammation. Importantly, such metabolic effects include transient alterations in lipids and peripheral insulin resistance (Feingold and Grunfeld, 1992). In healthy individuals these responses are necessary, forming part of an effective short-term response to infection and inflammation. However, the chronic elevation of certain cytokines observed during RA can have severe deleterious effects and

are capable of activating several physiological features known to increase the risk of developing atherosclerosis, including insulin resistance, dyslipidaemia and pro-oxidative stress (Feingold and Grunfeld, 1992, Guest et al., 2008, Prawan et al., 2009).

Pro-inflammatory cytokines (e.g. TNFα and IL-6) and chemokines (e.g. IL-8 and monocyte chemoattractant protein-1 (MCP-1)) are significantly elevated in RA patients (Ellingsen et al., 2001, Endo et al., 1991). Not only do these cytokines and chemokines play key roles in driving the synovial cell proliferation and chronic inflammation observed in RA, but they have also been demonstrated to play important roles in CVD pathogenesis. For example, IL-8 and MCP-1 have been suggested to play a pivotal role in atherosclerosis initiation by recruiting monocytes/macrophages to the vessel wall. Furthermore, plasma concentrations of IL-6 have been suggested to reflect the intensity of plaque inflammation and the vulnerability to rupture (Ito and Ikeda, 2003).

That systemic inflammation promotes CVD in RA is a hypothesis that has featured heavily in the literature on this subject (Sattar et al., 2003, Abou-Raya and Abou-Raya, 2006, Foster et al., 2010). However, despite several mechanisms having been proposed, our understanding of the exact pathomechanisms that link these diseases remains incomplete. It is unknown whether the immune profile of patients with RA that go on to develop premature CVD differs from that of other RA patients; that certain RA patients have an altered immune profile which renders them at high risk of CVD is one possibility.

1.5.4 Autoantigens and heat-shock proteins (HSPs)

Autoantibodies against self-antigens have long been associated with RA (*see section 1.3.1.2*). Accordingly, a wide range of autoantigens have been described by the reactivity of antibodies present in the serum of RA patients. The majority of suggested RA autoantigens fall into two distinct groups (Corrigall and Panayi, 2002). The first group encompasses autoantigens associated with the joint, such as collagen type II, human chondrocyte glycoprotein 39 and proteoglycans. The second group includes those proteins that are not associated with the joint. Such proteins are made up of (1) post-translationally altered proteins, such as citrullinated filaggrin; (2) highly conserved antigens with foreign (bacterial, viral) homologues, such as heat shock proteins (HSPs), in which the initiating antigenic stimulus may be through infection; (3) ubiquitous proteins, such as glucose-6-phosphate isomerase and HSPs released during times of stress.

It is well established that, within RA cohorts, traditional and non-traditional cardiovascular risk factors do not explain the observed increased CVD burden (Gonzalez et al., 2008), suggesting that alternative pathological processes may be responsible. One potential explanation is that autoantigens and autoantibodies previously considered as specific to the pathology of RA are also contributing to the development and progression of CVD.

In a study of 672 RA patients, it was determined that those positive for anti-cyclic citrullinated peptide (anti-CCP), a highly specific marker for RA, experienced more frequent ischaemic heart disease and had higher mortality rates. Furthermore, there was no association between anti-CCP and established CVD risk factors (Javier Lopez-Longo et al., 2009). However, whether citrullinated proteins, peptidyl arginine deiminase (the enzyme responsible for citrullination) or antibodies against citrullinated proteins play a role in the development of CVD in RA patients remains unknown.

Systemic lupus erythematosus (SLE) is an autoimmune rheumatic disease with many similarities to RA and for which the risk of fatal or debilitating CVD events is increased 10-fold (Hahn et al., 2007). Accordingly this disease may provide a valuable insight into RA pathogenesis. It has been shown that IgG autoantibodies which recognise high-density lipoproteins (HDLs), the major apolipoprotein constituent (Apo A-I) and also CRP exist in the sera of ~45% of SLE patients with high disease activity (O'Neill et al., 2010). As all three of these molecules play roles in the pathogenesis of CVD, the clinical implications of such autoantibodies must be considered. Several potential mechanisms by which these antibodies might contribute to CVD burden have been suggested (Hahn, 2010): (1) the binding of autoantibodies to HDL epitopes may remove the atheroprotective function of HDL; (2) soluble antibody-antigen complexes may attach to vascular tissue, fix complement, and trigger local inflammation; (3) antigen-antibody complexes are proinflammatory as immune recognition activates Fc expressing phagocytic cells; (4) antibody recognition is likely to interfere with normal metabolism of CRP, HDL and Apo A-I.

It should also be noted that HSPs have been implicated as potential autoantigens in both RA and CVD (Pockley, 2002, Bodman-Smith et al., 2003). In addition to being molecular chaperones, a significant element of the immune response to pathogens is directed towards HSP peptides (Kaufmann, 1990). Furthermore, as the name would suggest, increased synthesis of HSPs occurs when cells are exposed to stress. By increasing cellular HSP content, cells protect themselves from the uncontrolled protein unfolding that often

accompanies stress (Zuegel and Kaufmann, 1999). As a result of the chronic inflammatory nature of RA, cells are constantly under the influence of pro-inflammatory signals, so it is not surprising that several members of the HSP family have been reported to be elevated in the serum and synovium of RA patients (Huang et al., 2009, Schett et al., 1998).

Although the precise influence of HSPs on atherosclerosis remains unclear, an association between HSP expression and the induction of the inflammatory response that characterises the development of atherosclerosis has been demonstrated. In one particular study intensity of HSP expression was found positively to correlate with atherosclerosis severity. At the same time a localised enrichment of T cells, with an ability to recognise HSPs, was detected in the lesion (Kleindienst et al., 1993). Furthermore, immunisation of normocholesterolemic rabbits (Xu et al., 1992) and atherosclerosis model mice (Afek et al., 2000) with mycobacterial HSP65 was found to induce atherosclerosis.

Such studies raise the possibility that elevation of HSPs as a result of RA may lead to HSP overexpression at arterial sites vulnerable to atherosclerosis. Such expression may further promote a localised arterial inflammatory response, resulting in recruitment of immune cells to the vessel wall. This hypothesis is explored further in *chapter 3*.

1.5.5 Bacterial triggers

Despite conventional risk factors for CVD being well established, they can only account for 50 to 70% of CVD events within the general population (Haynes and Stanford, 2003), and even less in RA subjects (*figure 1.5*) (Gabriel, 2010). Recently, infection has been proposed as playing an aetiological role in CVD. In particular research efforts have concentrated on oral infection as an independent risk factor for CVD (Amar et al., 2003).

Several epidemiological studies have supported a link between periodontal disease (PD) and CVD (Hung et al., 2003, Destefano et al., 1993). CVD and PD are both major contributors to morbidity and mortality in developing and developed countries (Abou-Raya et al., 2007), therefore quantification of their association may provide significant improvements to public health (Slots, 1998). Furthermore, clinical studies support a significant association between RA and periodontal bacteria, with RA patients demonstrating a substantially higher incidence of PD when compared to age- and sex-matched controls (Mercado et al., 2001) (Rosenstein et al., 2004). Several anaerobic

periodontal pathogens have also been detected in both the synovial fluid and serum of patients with RA (Moen et al., 2006).

Periodontal diseases, including gingivitis and periodontitis, are serious infections that when left untreated lead to inflammation and tooth loss.

Two hypotheses to explain the link between infection and CVD and RA have been widely documented. Firstly, inflammation as a result of local or systemic bacterial infection may stimulate a systemic inflammatory response that, in turn, could potentiate the onset of atherosclerosis and/or RA (the systemic inflammation hypothesis). Alternatively, infection may lead to bacteraemia, with the potential for bacterial proliferation *per se* leading to atherosclerosis and/or RA (the bacteraemia hypothesis). The following two sections will consider these two hypotheses (*sections 1.5.5.1 and 1.5.5.2*). This is followed by suggestions as to how bacterial infection may help explain the link between RA and CVD (*section 1.5.5.3*).

1.5.5.1 Systemic inflammation hypothesis

Systemic inflammation as a result of either localised or systemic infection may contribute to both the development and maintenance of atherosclerosis through the activation of the immune and inflammatory cascade (Seymour et al., 2009).

As previously discussed, endothelial dysfunction has been popularised as the first step in atherosclerosis development (*section 1.4.3.2*) (Higashi et al., 2008) (Cai and Harrison, 2000). There is a well-established link between inflammation and endothelial dysfunction. Several studies have established that chronic bacterial infection impairs vasodilation in otherwise healthy subjects (Charakida et al., 2005). For example, Oshima *et al* (2005) demonstrated that PD impairs endothelium-dependent vasodilation but has no effect on endothelium independent vasodilation in hypertensive patients and subjects negative for all known cardiovascular risk (Oshima et al., 2005).

Another way in which bacteria can potentially up-regulate immune responses remotely is by inducing autoimmunity against host vasculature cells. Bacteria contain certain components that closely resemble host molecules. Thus, an immune response triggered by bacterial infection may lead to production of autoaggressive T cells or cross-reacting autoantibodies (Leinonen and Saikku, 2002). Heat shock proteins (Hsp) are an example of

autoimmunity-stimulating molecules, as they are widely conserved between both prokaryotes and eukaryotes. Bacterial Hsp60 shares much sequence and structural homology with human Hsp60 and may be a candidate for triggering an autoimmune response which could lead to, or maintain, atherosclerosis.

Other studies have implicated *Porphyromonas gingivalis*, the major periodontal pathogen, in playing a role in atherogenesis. Pollreisz *et al*. in an attempt to reveal the effect this pathogen has on systemic inflammation, infected murine monocytes and macrophages with *P. gingivalis (Pollreisz et al., 2009).* Infection with *P. gingivalis* significantly increased secretion of the pro-inflammatory cytokines IL-6 and TNF-α and stimulated monocyte migration. Since both recruitment of monocytes/macrophages and the secretion of pro-inflammatory cytokines are vital in the development of atherosclerosis, it is possible that *P. gingivalis* could be involved in the atherogenesis process.

Furthermore, fibrinogen, a co-factor in platelet aggregation expressed during the acute phase response (Leinonen and Saikku, 2002), could help explain why infection may contribute to atherosclerosis. Raised fibrinogen levels have been associated with atherosclerosis due to their effect upon platelet aggregation, leucocyte chemotaxis and vascular permeability (Levenson et al., 1995). Equally, it has recently been demonstrated that periodontal infections are associated with increased fibrinogen concentrations in patients with CVD (Buhlin et al., 2009). Taken together, these findings imply that raised fibrinogen levels, as a result of infection, could increase the risk of CVD development.

Bacteria may potentially alter host lipid metabolism, providing further support for the association between infection and CVD. Invasive bacteria are capable of altering host lipid metabolism in two ways. Firstly, the LPS from Gram-negative pathogens have been demonstrated to reduce high density lipoprotein (HDL) cholesterol (Grunfeld and Feingold, 1992). Secondly, during the acute phase response to infection, IL-1, IL-6 and TNFα release will increase triglyceride and cholesterol concentrations (Leinonen and Saikku, 2002). Thus, it is possible that chronic infection leads to an expression of cytokines which can create a pro-atherogenic lipid profile.

1.5.5.2 Bacteraemia hypothesis

If CVD development is found to be definitively associated with bacterial infection, a major hurdle to overcome will be in deciphering whether systemic inflammation or systemic infection is the causative link.

The hypothesis that a local or systemic infection may be capable of triggering a systemic inflammatory response which could potentiate the onset of atherosclerosis has been discussed previously (*section 1.5.5.1*). A similarly and equally plausible hypothesis is that subsequent to bacteraemia certain pathogens will be retained in the arterial vessel wall and trigger a localised inflammatory response associated with proteolytic and oxidative processes, eventually leading to atherosclerosis.

Definitive proof that bacteria can potentiate atherosclerosis requires experimental modelling. Murine models, following intravenous administration with the putative periodontal pathogen *Porphyromonas gingivalis,* have been found to go on to develop atherosclerosis (Li et al., 2002).

A significant body of literature has established elevated concentrations of CRP as a risk factor for CVD (de Ferranti and Rifai, 2002). Interestingly, elevated pathogen presence (either individually or as a cumulative pathogen burden) at the site of atherosclerosis has also been associated with an elevated concentration of CRP (Zhu et al., 2001a). This further implicates bacterial infection in the development of atherosclerosis.

Several pathogens in particular have been associated with the development of atherosclerosis. Viable *Chlamydia pneumoniae* (the human respiratory pathogen) has been commonly isolated from atherosclerotic plaques (Leinonen and Saikku, 2002). However, of the periodontal bacteria (*Porphyromonas gingivalis, Prevotella intermedia, Campylobacter rectus, Actinobacillus actinomycetemcomitans*), association with atherosclerotic plaques has almost exclusively been determined by the presence of bacterial DNA, and not by culture dependent techniques. A study conducted by Fiehn *et al* (2005) demonstrated that although DNA originating from periodontal pathogens could be isolated from atheromatous plaques, no viable oral bacteria could be isolated (Fiehn et al., 2005). These results could infer that if periodontal pathogens are in fact associated with CVD, it is either mediated by systemic inflammation or transient exposure to viable oral bacterial at the site of atherosclerosis. Alternatively, as periodontal pathogens are mostly anaerobic, they may bury deep in the arterial tissue, making cultivation difficult.

Arterial infection may potentiate the onset and maintenance of atherosclerosis through interaction with the innate immune system (Edfeldt et al., 2002). The expression of TLRs 2, 4, 5 and 9, all of which possess the ability to recognise bacterial ligands, have been found to be upregulated in atheromatous tissue. TLR stimulation will result in secretion of pro-inflammatory cytokines, which could potentiate atherosclerosis. Arterial cells are responsive to a wide range of ligands for TLRs 2, 4, 5 and 9. However, efforts to stimulate TLRs *in vitro* with homogenised atherosclerotic tissue have so far provided equivocal results, with ligands for only TLR2 only being stimulated by tissue homogenate (Erridge et al., 2008). This observation may be the result of bacterial PAMPs being present in atheromatous tissue only transiently.

If systemic bacterial infection was a risk factor for CVD, it could potentially allow intervention using conventional antibiotic treatment. Antibiotic therapy with azithromycin, a broad-spectrum antibiotic suitable for treatment of *Chlamydia pneumoniae* and a range of periodontal pathogens in patients with history of myocardial infarction, did not significantly reduce any of the outcomes of CVD (myocardial infarction, angina and death) (O'Connor et al., 2003). These results do not dismiss the possibility that bacteria play a role in the pathogenesis of CVD. As previously mentioned, bacteria may exert their pathogenic effect only transiently, thus triggering an immune cascade capable of potentiating atherosclerosis.

Antibiotics appropriate for the treatment of anaerobic pathogens, such as clarithromycin, ornidazole and levofloxacin, have been demonstrated to be effective against RA. In one study, a significantly greater proportion of patients administered with 1000mg ornidazole met the 20% improvement criteria set by the American College of Rheumatology when compared to patients who were administered a placebo (Ogrendik, 2006). However antibiotics, most notably clarithromycin, have been demonstrated to possess immunomodulatory functions and can inhibit TNFα and IL-1 synthesis. As several biologic immunomodulatory drugs are currently used in the treatment of RA, it is possible that the mechanism of action of these antibiotics in the treatment of RA is not through their effect on bacterial viability.

1.5.5.3 Bacteria: a candidate to help explain the association between RA and CVD

A high frequency of bacterial infection complicating RA has been described in case reports. It has been suggested that this occurs due to both underlying disease complications as well as the side effects of immunosuppressive RA therapeutics (Smitten et al., 2008). Data from longitudinal cohort studies have demonstrated that patients with RA are indeed at increased risk of developing infections compared to non-RA subjects (Doran et al., 2002) and that higher disease activity was associated with a higher probability of developing infections (Au et al., 2011). Furthermore, it has been found that the elevated risk of infection leading to hospitalisation is associated with disease-modifying anti-rheumatic drug (DMARD) use (Smitten et al., 2008).

As previously discussed, RA is generally considered to have a multifactorial aetiology, with susceptibility presumed to be principally due to genetics combined with environmental triggers, of which bacterial infection is considered the most prominent. Most autoimmune diseases, including RA, are associated with specific HLA alleles. Greater than 90% of RA patients possess HLA-DR1 (Ebringer and Wilson, 2000). It has been suggested that the involvement of HLA antigens in the pathogenesis of RA is due to molecular mimicry between certain bacterial antigens and HLA antigens (Wilson et al., 2000).

Considering all the evidence, it is plausible to hypothesise that RA increases a patient's susceptibility to infection by bacterial pathogens. Furthermore, the systemic inflammatory state present in RA may select for certain bacterial pathogens. If correct, these could potentially increase the risk of pro-atherogenic bacteria entering the circulation of RA patients and help to explain why RA patients experience increased CVD burden.

1.6 Hypothesis and aims

- It has been proposed that atherosclerosis begins in the aortic adventitia and progresses to the intima (the so-called 'outside-in' hypothesis), but this tissue has rarely been examined in the context of CVD. Given that RA is a systemic inflammatory condition, it is plausible that this inflammation affects the vascular environment.

    - ❖ *Is the aortic adventitia immunogenic? Does the inflammatory mileu of the aortic adventitia differ between CVD and RA+CVD patients?*

- Recently, infection has been proposed as playing an aetiological role in CVD. Owing to the pathological profile associated with RA, it is possible that patients are more susceptible to potentially pro-atherogenic bacterial infection.

    - ❖ *Are bacteria present in the aortic adventitia, and do differences in the bacterial profile exist between CVD and RA+CVD patients?*

- Systemic inflammation in RA has been proposed as a major contributor to CVD co-morbidity. However, the immune profile of RA+CVD has not been characterised.

    - ❖ *Does the systemic immune profile differ between RA, CVD and RA+CVD patients, and could any differences contribute to increased CVD burden in RA?*

2 Materials and Methods

2.1 Immunohistochemistry

2.1.1 Human subjects

Patients were enrolled at a cardiac surgery centre (Feiring Heart Clinic, Feiring) and were matched at the group level for age and sex. Patients were screened for the presence of RA according to the ACR and EULAR classification criteria (*table 1.2*). The patients were evaluated preoperatively by a single rheumatologist (Ivana Hollan, Department of Rheumatology, Hospital for Rheumatic Diseases, Lillehammer, Norway) to verify the rheumatologic diagnosis and to assess disease severity and activity; blood samples were collected at that time. Inclusion criteria for the RA group were age older than 18 years and a confirmed diagnosis of RA according to accepted criteria (Aletaha et al., 2010).

2.1.2 Aortic adventitia paraffin sections

The human aortic adventitia samples were from the Fering Heart Biopsy Study. Aortic specimens were obtained from tissue that is routinely removed during CABG surgery. Subsequently, part of the adventitia covered by the epicardium was removed from the ventral part of the ascending aorta, and 1-3 punch holes were made through the vessel wall for proximal aortocoronary anastomoses in the same area. To avoid thromboembolic complications, the specimens were obtained from areas with less pronounced gross signs of atherosclerosis. A portion was fixed in formalin and then embedded in paraffin. All the patients gave written informed consent. Regional Ethics committee for Medical Research Norway approved the Feiring Heart Biopsy study.

2.1.3 Haematoxylin and eosin staining

Tissue sections were deparaffinised in two changes of absolute xylene for 10 minutes each followed by gradient rehydration in two changes of 100%, 90% and 70% ethanol (VWR, Lutterworth, UK) for three minutes each. Sections were briefly rinsed in distilled water followed by immersion in Harris haematoxylin for five minutes. Sections were then rinsed in running tap water for three minutes followed by differentiation in 1% acid alcohol for 30 seconds. The sections were then rapidly transferred into running water and rinsed for one minute and then blued in Scott's tap water substitute for two minutes. The sections were rinsed again in running tap water and counterstained in eosin for one minute. Following a

rapid rinse in running tap water sections were dehydrated through an alcohol gradient of 70%, 90% and 100% for three minutes each. Sections were then transferred into absolute xylene for 10 minutes and then mounted with DPX (Sigma-Aldrich, Poole, UK).

2.1.4 Optimisation

Aortic adventitial tissue was scarce and prone to high levels of background staining. As a result, immunohistochemistry staining was first established in human tonsil or RA synovium tissue. Each antibody was optimised by altering elements of antigen retrieval, blocking systems and secondary antibodies. Details of primary antibodies, retrieval systems and secondary antibodies are listed (*table 2.1*) and the final optimised protocol for each antibody is outlined in *table2.2*.

Table 2.1 Details of antibodies, antigen retrieval and immunohistochemistry system used in staining of human paraffin sections

Antibody	**Code**	**Source**	**Antigen Retrieval**	**Staining System**
Heat shock Protein 60	ab1819	Abcam	Trypsin, 37^{o}C, 15 min	Horse anti-mouse ImmPRESS
Heat shock Protein 47	ab77609	Abcam	MW* 700w, 15 min	Horse-anti mouse ImmPRESS
CD21	M0784	DAKO Cytomation	Trypsin, 37^{o}C, 30 min	ABC kit
Calprotecin S100A9	NA	NA	MW* 700w 15 min	Horse anti-mouse ImmPRESS
TNFα	ab1793	Abcam	MW* 700w 15 minutes	Horse anti-mouse ImmPRESS
CD68	MO876	DAKO Cytomation	MW* 700w 15 minutes	ABC

2.1.5 Deparaffination and hydration

Paraffin sections were cut (kindly performed by Ivana Hollan, Lillehammer, Norway) to a thickness of 4-5 µm and then mounted on electrostatically coated microscope slides (VWR). Deparaffination of the sections was carried out by initially incubating the slides at 65^{o}C for 45 minutes to soften the paraffin wax. Sections were then deparaffinised by immersion in xylene for 10 minutes. Following this, sections were hydrated by immersion in 100%, 90% and 70% ethanol for six minutes each followed by immersion in distilled water for five minutes. The endogenous peroxidise activity was blocked by immersing sections for 30 minutes in 0.5% hydrogen peroxidise (Sigma-Aldrich) (*Appendix 2*) at room temperature.

2.1.6 Antigen retrieval

Two different antigen retrieval methods (trypsin or citrate) were used, depending on which antibody was being used. For trypsin retrieval each section was covered with trypsin (Vector Laboratories, Peterboroughm UK) working solution (*appendix 2*) and incubated at 37^0C for 15 minutes to allow enzymatic activity and then left at room temperature for five minutes. The sections were then washed in TBS buffer (Sigma-Aldrich) (*appendix 2*) for a further five minutes. Antigen retrieval by citrate buffer (Vector Laboratories) (*appendix 2*) was performed by microwaving 600ml of citrate buffer for five minutes at 700 watts in order to bring the solution to the boil. The slides were then added to the boiling solution and microwaved for a further eight minutes at 700 watts. The sections were then left to cool for 15 minutes in the citrate buffer.

2.1.7 Non-specific binding block and primary antibody

Following antigen retrieval, and prior to primary antibody incubation, a non-specific binding block was performed for every section. Non-specific binding was blocked by incubating sections with 5% normal horse serum (Vector Laboratories) for 30 minutes at room temperature. This was followed by incubation with primary antibody overnight at 4^oC. The optimal antibody concentration, and any alterations to the steps outlined here are listed in *table 2.1* and *2.2*. Primary antibodies were diluted in TBS buffer (*appendix 2*) with 2.5% horse serum and 2.5% human serum.

2.1.8 Secondary antibody and mounting

Following incubation with primary antibody, sections were brought back to room temperature and washed twice in TBS buffer. Each section was then incubated for 30 minutes with the appropriate secondary antibody. The most commonly used secondary antibody (*table 2.2*) was an antibody coupled to peroxidise molecules (ImmPRESS kit, Vector laboratories). Sections were then washed twice in TBS buffer. The staining was then developed with DAB (Diaminobenzidine: Vector Laboratories) (*appendix 2*) until positive proteins were visualised but background staining was not evident. Stain development was stopped by immersing the sections in TBS buffer and then running under water. Sections were then counter stained in haematoxylin and again immersed in running water. The sections were then dehydrated by passing through 70%, 90% and 100% ethanol

for three minutes each and then immersing in xylene for 10 minutes. A cover slip was then placed over each section using DPX (Di-N-Butyle Phthalate in xylene) (Sigma-Aldrich).

2.1.9 Major staining steps for each antibody

Immunohistochemistry protocols were optimised for each primary antibody (*section 2.1.4*). All optimisation was performed in paraffin-embedded human tonsil and if available rheumatoid synovium sections. Antigen retrieval, antibody dilutions and staining systems were thus specific for each staining. The major steps for each staining are highlighted in *table 2.2* below.

Table 2.2 Sequence of major immunohistochemistry steps performed

Antibody dilution	**Isotype control**	**Order of primary steps**	**Positive tissue**
CD21 1/13	Monoclonal mouse IgG1	a) Antigen retrieval in 0.1% trypsin b) Endogenous peroxidise block c) Non-specific block with 10% horse serum d) Primary antibody incubation in 2% goat serum in PBS at RT for 90 minutes e) Secondary antibody incubation with biotinylated goat anti-mouse immunoglobulin (1:200 dilution) e) avidin/biotin complex f) Developed with impact DAB	Tonsil
TNFα 1/25	Monoclonal mouse IgG1	a) Heat retrieval with citrate b) Non-specific block with 2.5% horse serum c) Overnight primary antibody incubation	Tonsil

		at 4°C	
		d) Endogenous peroxidise block	
		e) Incubation with peroxidise linked secondary antibody	
		f) Developed with impact DAB	
HSP60 1/50	Monoclonal mouse IgG1	a) Antigen retrieval in 0.1% trypsin b) Non-specific block with 2.5% horse serum c) Overnight primary antibody incubation at 4°C d) Endogenous peroxidise block e) Incubation with peroxidise linked secondary antibody f) Developed with impact DAB	Tonsil
HSP47 1/50	Monoclonal mouse IgG1	a) Heat retrieval with citrate b) Non-specific block with 2.5% horse serum c) Overnight primary antibody incubation at 4°C d) Endogenous peroxidise block e) Incubation with peroxidise linked secondary antibody f) Developed with impact DAB	RA synovium
Calprotectin 1/100	Monoclonal mouse IgG1	a) Heat retrieval with citrate b) Non-specific block with 2.5% horse serum horse serum c) Overnight primary antibody incubation at 4°C	Tonsil

		d)	Endogenous peroxidise block	
		e)	Incubation with peroxidise linked secondary antibody	
		f)	Developed with impact DAB	
CD68 1/100	Monoclonal mouse IgG1	a)	Endogenous peroxidise block	RA synovium
		b)	Antigen retrieval with citrate	
		c)	Incubation with non-specific binding block plus avidin	
		d)	Incubation with primary antibody and biotin block	
		e)	Secondary antibody incubation with biotinylated pan-specific horse immunoglobulin	
		f)	Incubation with avidin/biotin complex	
		g)	Developed with impact DAB	

2.1.10 Analytical procedure and data collection

The aortic adventitia is composed primarily of adipocytes, mesothelial, endothelial and smooth muscle cells. There was also a varying extent of inflammatory infiltrate detectable in most sections. Stained sections were analysed in random order while blinded to experimental data. Sections stained by immunohistochemistry were analysed by light microscopy (Olympus IX51, Essex, UK). When applicable, the extent of staining was measured in each cell type (mesothelial, endothelial, smooth muscle and inflammatory infiltrate). The percentage of each cell type expressing the stained protein was then calculated and assigned a value (*table 2.3*).

0%	**0**
<10%	**1**
10-25%	**2**
>25%	**3**

Table 2.3 Scoring system for immunohistochemistry. Percentage represents number of cells stained.

2.1.11 Measuring surface area of section

Total inflammation in the aortic adventitia was calculated using Cell^D software (Olympus). Each section was first imaged by light microscopy at 100x magnification (Olympus IX51). Sequential, non-overlapping images were captured spanning the entire surface area of each section. The surface area of each picture was then measured using the Cell^D measurement package, thus allowing total section area to be expressed in μm^2. Sites of inflammatory cell infiltrate were visually identified and measured in an identical manner. Inflammatory cell infiltrate was then expressed as a percentage of the total section area.

2.1.12 Statistical analysis

Data was analysed using the most appropriate statistical test. All results are accompanied with a description of the statistical test used. When comparing two groups of normally distributed data a student's t-test was used to determine statistical significance. If the data was not consistent with normal distribution, differences in quantitative variables were analysed for with a non-parametric Mann Whitney U test. In both cases a *P* value of <0.05 was considered significant. When applicable, graphs are displayed as dot plots to clearly illustrate the distribution of scores. Statistical analysis was performed using either SPSS version 19 for PC (IBM Software, New York, USA) or GraphPad Prism version 4 for PC (GraphPad Software, Calofornia, USA).

2.2 Bacterial detection and investigation

2.2.1 Aortic adventitia frozen sections

Aortic tissue was collected as described in *section 2.1.*2. A portion was snap frozen in liquid nitrogen upon removal and then stored at -80^{o}C until required.

2.2.2 DNA extraction from tissue samples

At all stages a strictly aseptic procedure was used. Tissue samples were lysed by incubation for 1 hour at 55^{o}C with 15µl Proteinase K solution (20 mg/ml) (Sigma-Aldrich). Tissue samples were then homogenised on dry ice in 1.0ml TRIzol (Invitrogen, Paisley, UK) using a TissueRuptor kit and sterile probe (Qiagen Crawley, UK). 200µl of chloroform (Sigma-Aldrich) was added, followed by incubation at room temperature for 15 minutes then centrifugation at 12.0 x *g* for 15 minutes at 4^{o}C. The aqueous phase was removed, and the remaining interphase and organic phases were resuspended in 300µl of 100% ethanol. The sample was centrifuged at 12.0 x *g* for 5 minutes to form a DNA pellet. The pellet was washed twice in 1ml 0.1M sodium citrate (Sigma-Aldrich) before being resuspended in 1ml 75% ethanol. This was then centrifuged at 12.0 x *g* for five minutes at room temperature and the supernatant removed. The remaining pellet was dissolved in 100µl of sterile water (Qiagen), and the DNA containing supernatant removed and stored at -20^{o}C. Control samples which replaced tissue sample with sterile water were run in parallel to monitor for sterility of reagents and apparatus (see *section 2.2.7*).

2.2.3. PCR amplification of 16S ribosomal DNA

PCR amplification of 16S rRNA gene was performed using well established universal eubacterial primers (Marchesi et al., 1998): 5'- CAGGCCTAACACATGCAAGTC-3' (63f) and reverse; 5'- GGGCGGWGTGTACAAGGC-3' (1387r). The selected primers supported amplification of a 1324-bp segment of the 16SrRNA gene sequence. PCR was performed in a total volume of 50µl containing 100ng of genomic DNA 1 x GoTaq® PCR buffer (Promega, Southampton, UK) 1.25 U Go*Taq*® polymerase (Promega) 1.5 mM $MgCl_2$, 0.2 mM dNTPs (New England Biolabs, Hitchin, UK) and each primer at a concentration of 0.2 µM. Thermal cycling was conducted using a MyCycler (Bio-Rad Laboratories, Hemel Hempstead, UK) and comprised one cycle of 95°C for 2 minutes,

followed by 35 cycles of 95°C for 1 minute, 55°C for 1 minute and 72°C for 1.5 minutes, followed by a final extension cycle at 72°C for 10 minutes. Each PCR reaction contained a negative control with sterile molecular grade water (Qiagen) replacing DNA; the positive control contained 100 ng DNA isolated from cat plaque (kindly donated by Sanne Dolieslager). PCR products were then size separated by electrophoresis on a 2% agarose gel (Sigma-Aldrich) DNA bands were stained with ethidium bromide and visualised and photographed (Gel Doc XR+; Biorad).

2.2.4 Cloning and restriction digestion

PCR products were ligated into the StrataClone® PCR Cloning Vector pSC-A-amp/kan (Agilent Technologies, Wokingham, UK) followed by transformation into competent *E. coli* cells (Invitrogen) by heat-shock at 42^{o}C. Transformed cells were grown overnight at 37^{o}C on Luria-Bertani plates supplemented with 100mg/ml ampicillin (Invitrogen). Complete insertion was verified by PCR with M13 forward and reverse primers (Invitrogen). The 16S rRNA gene insert was then subjected to restriction digestion. 0.5µl of each PCR reaction was digested in a total volume of 18µl containing 0.2µl of either Rsa*I* or *Mnl*I (Thermo Fisher Scientific, Loughborough, UK) at 37^{o}C for one hour. Digestions were spun down and run on a 2% agarose gel. Clones were initially segregated into restriction fragment length polymorphism (RFLP) groups based on their *Rsa*I profile, and then further discrimination was made based on the *Mnl*I digestion profile.

2.2.5 Sequencing

The 16S rRNA gene insert of a single representative clone from each RFLP group was sequenced. Sequencing was performed using the SequiTherm EXCEL™ II DNA Sequencing Kit (Cambio, Cambridge, UK) and IRD800-labelled 357f sequencing primer (5'-CTCCTACGGGAGGCAGCAG-3'). The following cycling parameters were used: (i) initial denaturation at 95^{o}C for 30 seconds; (ii) 10 seconds at 95^{o}C and 30 seconds at 70^{o}C for 15 cycles. Formamide loading dye (6µl) was added to each reaction mixture after thermal cycling and 1.5µl of each reaction mixture was run on a LI-COR Gene ReadIR 4200S automated DNA sequencing system (MWG Biotech, Milton Keynes, UK).

2.2.6 DNA sequence analysis

Sequencing data were converted to FASTA format and compared with bacterial 16S rRNA gene sequences from the EMBL and GenBank sequence databases using the BLAST program (Altschul et al., 1997). The program was run through the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST). Clone sequences that demonstrated at least 97% identity with a known sequence from the database were considered to be the same species as the matching sequence with the highest score. Sequences with less than 97% identity were classified as potentially novel phylotypes.

2.2.7 Contamination control

Owing to the sensitivity of 16S rRNA gene sequencing it was vital to control for the introduction of contamination at every stage. A negative control was processed in parallel with every tissue sample. Each control was treated in exactly the same manner as the sample with the exception that sterile nuclease-free water was used instead of tissue. To further ensure experimental sterility, each reagent used in the DNA extraction protocol was initially tested for the presence of possible bacterial DNA contamination. Aliquots of TRIzol, chloroform, 100% ethanol, 75% ethanol, 0.1M sodium citrate (Sigma-Aldrich) isopropanol and DEPC treated water were allowed to completely evaporate from a 1.5ml eppendorf tube in a class II laminar flow hood and then resuspended in sterile nuclease-free water (Qiagen). Each sample was then subjected to Proteinase K digestion (Sigma-Aldrich) followed by 16S rRNA gene amplification to determine if any contamination was present. Each sample was diluted in mastermix (10, 4 and 1%) (Promega, UK) as excessive DNA concentrations can inhibit the PCR reaction by preventing product formation. Samples were then subjected to PCR using exactly the same primers and conditions highlighted in *section 2.2.3*.

2.3 *In vitro* investigation of *Methylobacterium sp.*

2.3.1 Culture of *Methylobacterium mesophilicum* and *Methylobacterium oryzae*

Methylobacterium mesophilicum (1708) and *Methylobacterium oryzae* (18207) type strains were obtained from the DSMZ culture collection (DSMZ, Braunschweig, Germany). Both strains were grown in nutrient agar (5g peptone, 3g meat extract, 15g agar for solid media, made up to 1000ml with distilled water) supplemented with 1% (v/v) methanol. A pH of 7.0 was maintained by the addition of hydrochloric acid (10mM). Strains were grown on solid agar in petri dishes or in 10ml tubes in a shaking incubator at 30^{o}C.

2.3.2 Scanning electron microscopy (SEM)

Planktonic bacterial cells were examined by SEM, as previously described (Erlandsen et al., 2004). Briefly, bacterial cells were centrifuged (400 x *g*) and resuspended in fixative (2% para-formaldehyde, 2% gluteraldehyde and 0.15M sodium cacodylate). The fixative was then replaced with 0.15M sodium cacodylate buffer, and samples washed for 3 x 5 minutes. A 1% (w/v) osmium tetroxide (OsO4) solution was then added to an equal volume of 0.15M sodium cacodylate buffer for 1 hour. Samples were then rinsed three times with ddH_2O for 10 minutes. 0.5% (v/v) aqueous uranyl acetate was then added, and samples were incubated in the dark for 30 minutes at room temperature. Samples were then dehydrated in an ascending ethanol series, followed by fixing in hexamethyldisilazane (HMDS) (Carbosynth, Berkshire, UK) for 2 x 5 minutes in separate containers. The samples were then placed in a dessicator overnight. The fixed and dried bacterial samples were sputter-coated with gold/paladium and viewed under a JEOL JSM-6400 scanning electron microscope (JEOL, Herts, UK).

2.3.3 SVEC-10 murine cell culture

A murine lymphoid vascular endothelial cell line (SVEC-10) (Oconnell and Edidin, 1990) immortalized with simian virus 40 (SV40) was grown in T-75 flasks at 37^{o}C in 5% CO_2 in Dulbecco's medium (Sigma-Aldrich) supplemented with 100mM sodium pyruvate (Sigma-Aldrich), 200mM L-Glutamine (Life Technologies, Paisley, UK), 10,000 units Pen-strep (Life Technologies) and 10% foetal calf serum (Sigma-Aldrich). Cells were cultured until

90% confluence as determined by light microscopy and then split. Growth media was removed then the cell monolayer was gently washed twice with sterile 1 x PBS (Sigma-Aldrich). The monolayer was then trypsinised by the addition of 5ml of sterile 1x 0.5% trypsin EDTA (Sigma-Aldrich). This was then incubated at 37^{o}C for three minutes or until de-attachment of the endothelial cells was visible by light microscopy. The flask was then shaken firmly a couple of times and 10ml of complete media added to stop the trypsinisation reaction. Cells were then removed and placed into a 15ml tube and centrifuged at 1500 rpm for 10 minutes. The cells were then washed in fresh media and approximately 15% of the cells were used to seed a new T-75 flask.

2.3.4 RAW 264.7 murine cell culture

A murine leukaemic monocyte macrophage cell line (RAW 264.7) was grown in T-75 flasks at 37^{o}C in 5% CO_2 in RPMI-1640 medium (Sigma-Aldrich) supplemented with 200mM L-Glutamine (Life Technologies), 10,000 units Pen-strep (Life Technologies) and 10% foetal calf serum (Sigma-Aldrich). Cells were cultured until 90% confluence as determined by light microscopy and then split. Growth media was removed and the cell monolayer was gently washed twice with sterile 1 x PBS (Sigma-Aldrich). PBS was removed and 10ml of fresh complete media added. The cells were then harvested using a cell scraper (Corning, USA). The scraped cells in media were removed and placed into a 15ml tube and centrifuged at 1500 rpm for 10 minutes. The cells were then washed in fresh media and approximately 15% of the cells were used to seed a new T-75 flask.

2.3.5 Isolation and culture of CD14^{+} monocytes by EasySep® positive selection

Human buffy coats (the fraction of a total blood following gradient centrifugation that primarily contains white blood cells and platelets) were graciously provided by the Western Infirmary (Glasgow, UK). The buffy coat was mixed in a 1:1 ratio with room temperature Dulbecco's phosphate buffered saline (DPBS) without $CaCl_2$ and $MgCl_2$ (Sigma-Aldrich). 3ml of Histopaque-1077 (Sigma-Aldrich) was added to 15ml falcon tubes and allowed to reach room temperature. 8ml of the diluted buffy coat was gently overlaid onto the histopaque-1077. Tubes were spun at 400 x *g* with no brake for 30 minutes at room temperature. Peripheral blood mononuclear cells (PBMCs) were carefully

collected from the opaque interphase of PBS/Ficoll with a Pasteur pipette and transferred to a 50ml centrifuge tube. The total volume was brought to 50ml by addition of DPBS modified without $CaCl_2$ and $MgCl_2$ and mixed by gentle inversion. Cells were spun at 240 x *g* for 10 minutes at room temperature with full break applied. The supernatant was decanted taking great care not to lose any of the PBMC pellet. The PBMC pellet was resuspended in 10ml of cell separation media (Ca^{2+}/Mg^{2+} free PBS containing 2% FBS and 1mM sterile EDTA) and mixed by gentle trituration with a Pasteur pipette. At this stage 50µl of PBMCs were removed for cell counting. PBMCSs were then spun down at 240 x *g* for 10 minutes at room temperature with full break applied and resuspended in a volume of cell separation media to obtain $1x10^8$ PBMCs per ml.

Once PBMCs had been isolated $CD14^+$ cells were purified by EasySep® selection kit (StemCell Technologies, Grenoble, France) as per the manufacturer's instructions. This procedure supports processing of up to 2.5 x 10^8 PBMCs per preparation. The cell suspension at a concentration of 1 x 10^8 PBMCs/ml of separation was transferred into a 12 x 75mm polystyrene tube (Becton Dickson, UK) which fits into the EasySep®Magnet. PBMCs were labelled at room temperature for 15 minutes by addition of EasySep®Positive selection cocktail (StemCell Technologies) at 75µl/ml containing bispecific Tetrameric Antibody Complexes (TAC) directed against CD14 and dextran. Following this, dextran-coated EasySep®Magnetic Nanoparticles (StemCell Technologies) were incubated with the cell suspension at 50µl/ml at room temperature for 10 minutes to allow cross-linking with the labelled cells. The cell suspension was then placed into the EasySep®Magnet without the lid for 5 minutes. The TAC $^+$ nanoparticle labelled $CD14^+$ cells remain in the tube held by the magnetic field. $CD14^+$ cells were enriched by pouring off the material not held by the magnet. This process of enrichment was repeated three times adding fresh separation media each time. Finally, $CD14^+$ enriched cells were harvested by removing the tube from the magnet.

$CD14^+$ monocytes were then cultured for seven days at 37^oC in 5% CO_2 in RPMI-1640 medium (Sigma-Aldrich) supplemented with 200mM L-Glutamine (Life Technologies), 10,000 units Pen-strep (Life Technologies) and 10% foetal calf serum (Sigma-Aldrich) as well as 15ng/ml human M-CSF (Peprotech, London, UK). Cells were replenished in fresh media following three days of culture.

2.3.6 Purity evaluation of $CD14^+$ cells by FACS analysis

Flow cytometry was used to determine the final purity of the $CD14^+$ monocytes obtained by the StemCell method. Cell populations were analysed on a FACS caliber (BD Biosciences, Oxford, UK) with FlowJo software (Treestar, Ashland, USA) to evaluate pre and post separation cells. The $CD14^+$ purified cells, and $CD14^-$ cells (population not retained by the EasySep®Magnet) were stained for different cell surface antigens; CD3-FITC (BD Biosciences), CD19-APC (BD Biosciences) and CD14-PE (BD Biosciences). 1 x 10^6 cells from each population were transferred into FACS tubes and suspended and washed in 1ml of FACS buffer. The supernatant was then aspirated and cells were resuspended in 50µl of FACS buffer. 2.5µl of each antibody was added to the cell suspension followed by a 15 minute incubation in the dark at 4°C. 500µl of FACS buffer was then added and FACS tubes were centrifuged at 421 x *g* for 5 minutes and the supernatant removed. Cells were then fixed for later analysis by incubation with 250µl of Cytofix/Cytoperm (BD Biosciences) for 15 minutes at 4°C. The cell suspension was then spun down at 400 x *g* for 5 minutes and the pellet was resuspended in 250µl 1 x permwash (BD Biosciences). This was then centrifuged at 400 x *g* for 5 minutes and finally 350µl of FACS buffer was added. Cells were then ready for acquisition.

2.3.7 *Methylobacterium* challenge and RNA isolation

Murine cell lines and human primary macrophages were cultured as described previously (*section 2.3.3 – 2.3.5*). 1 x 10^6 cells were seeded into each well of a 6-well plate and grown until they reached 100% (endothelial) and 70% (macrophage) confluence. Cells were then challenged with exponentially growing *Methylobacterium mesophilicum* or *Methylobacterium oryzae* at an MOI of 200. *Methylobacterium* sp. were spun down and resuspended in either Dulbecco's media or RPMI media prior to inoculation. Controls were included which were treated in exactly the same manner as above with the exception of no bacteria being inoculated into the flask. Cells were harvested at 4 hours and 8 hours post infection.

Total cellular RNA purification was carried out using an RNeasy Kit (Qiagen) according to the manufacturer's instructions. Briefly 600µl of buffer RLT was added to each well and allowed to incubate at room temperature for 2 minutes in order for complete cell lysis to occur. Lysate was collected and 600µl of 70% ethanol added. The sample was then mixed

and the entire contents loaded onto an RNeasy spin column and centrifuged at 8000 x *g* for 15 seconds and the flow through discarded. DNase digestion was then performed directly on the column to ensure no residual genomic DNA contamination. Firstly the column was washed by spinning down for 15 seconds at 8000 x *g* with 350µl buffer RW. 10µl DNase I was mixed with 70µl buffer RDD. The solution was then pipetted directly onto the membrane of the spin column and incubated at room temperature for 15 minutes, following which the reaction was then stopped by the addition of 350µl buffer RW1 to the column. The column was then centrifuged at 8000 x *g* for 15 seconds. 500µl of buffer RPE was then added to the column and also spun for 15 seconds at 8000 x *g* followed by disposal of the flow through. As buffer RPE contains ethanol, an additional step to ensure no residual ethanol was present was carried out. This involved placing the spin column in a new collection tube and centrifuging for 1 min at full speed. The RNA was then eluted into a sterile 1.5ml tube by placing 30µl of RNAse free water (Qiagen) onto the spin column and centrifuging for 1 minute at 8000 x *g*.

2.3.8 cDNA synthesis

Following RNA extraction the resulting concentration and purity was measured by NanoDrop spectrophotometry (Thermo Scientific). RNA was regarded as sufficiently pure if the A_{260}:A_{280} was within the range of 1.7-2.1. 500ng of RNA was subjected to reverse transcription. As recommended for use with TaqMan Low Density Arrays (TLDA) (Applied Biosystems, Paisley, UK), cDNA synthesis was performed using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, UK). For each reaction the following reagents were added to a 200µl PCR tube on ice; 10µl 2x RT buffer, 1µl 20x RT enzyme mix; RNA (up to 9µl). The total volume was then brought up to 20µl with the addition of nuclease-free water (Qiagen). For each sample a non-RT control was included. For this, all stages remained the same with the exception of no 20x RT enzyme mix being added. Each tube was briefly centrifuged to eliminate any air bubbles. The tubes were then loaded onto a MyCycler Personal Thermal Cycler (Bio-Rad) and subjected to the following cycling conditions; 37°C for 60 minutes followed by 95°C for 5 minutes. DNA contamination was analysed by standard PCR for murine or human GAPDH. cDNA samples were stored at -20°C until needed.

2.3.9 TaqMan mRNA analysis by RT-QPCR

Gene expression was quantified using TaqMan Low Density Arrays (TLDA) (Applied Biosystems). A TLDA array consists of a 384 well micro fluidic card that supports 384 simultaneous RT-PCR reactions for targets that are pre-loaded onto each well. A standard 384-well Mouse Immune TLDA array or Human Immune TLDA array was used. Each card contained 96 different Taqman gene expression targets and supported four parallel runs. *Appendix 6* lists the 96 genes that were included in this study, and provides a brief explanation of the corresponding protein function. An average of 400ng of DNase-treated cDNA was used in each sample. 100µl of TaqMan gene expression mastermix (Applied Biosystems) and 100µl of cDNA in sterile water (Qiagen) was first mixed and then loaded onto the card. This mixture was equally divided over two loading ports (100µl) each supplying 48 genes of interest. The arrays were then centrifuged twice at room temperature for 2 minutes at 1300 rpm (Beckman Coulter, High Wycombe, UK, L-80 ultracentrifuge) to ensure equal distribution of the sample. Following this the card was sealed to prevent sample exchange between wells. Real time qPCR was performed using an Applied Biosystems 7900HT Fast Real-Time PCR System. The following thermal conditions were employed: 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 30 seconds at 97°C and 1 minute at 59.7°C.

2.3.10 TLDA analysis

Gene expression was quantified using the comparative threshold (C_T) method as suggested by the manufacturer (User Bulletin 2, Applied Biosystems). This technique utilises the formula $2^{-\Delta\Delta CT}$ in order to calculate the relative number of gene transcripts. The C_T value represents the PCR cycle at which the amplified gene target reaches a defined threshold. 40 PCR cycles were performed; accordingly the C_T values can range from 0 to 40. C_T values for each immunological molecule were calculated. If a C_T value was greater than 34, the datum was dismissed, as high C_T values invariably give rise to poor precision and reliability. C_T values were calculated for the *Methylobacterium* sp.-stimulated sample and the non-stimulated control sample. The C_T of several endogenous control genes was also calculated. GAPDH was calculated to be the most reliable endogenous control. The ΔC_T value was calculated [$\Delta C_T = C_T$ (target gene) − C_T(GAPDH)]. This was performed for each gene in both the *Methylobacterium* sp.-stimulated sample and the non-stimulated control sample. The relative expression of each gene was then calculated with the formula

$\Delta\Delta C_T = \Delta C_T$(positive sample) − ΔC_T(control sample). This was then expressed in terms of fold change relative to the control sample with the formula [fold change = $2^{-\Delta\Delta CT}$]. For down-regulated genes (value less than 1) the negative inverse of the value was taken. This allowed down-regulation to be expressed as a negative value.

2.3.11 Ingenuity pathways analysis

The 'Core Analysis' function included in the IPA (Ingenuity System Inc, Redwood City, USA) software package was used to identify and interpret the principal biological pathways and processes associated with human and murine macrophages following *Methylobacterium* sp., infection. All identifier types (different gene transcripts) with a C_T value of less than 34 were included in this study. Identifier types were first normalised to GAPDH and then expressed as fold change relative to non-infected macrophages. Both up- and down- regulated identifier types were defined as value parameters for analysis. A working file was generated which contained one column including all the original gene IDs (Unigene nomenclature) and another column indicating fold change ± SD relative to control macrophages. Following the analysis, generated canonical pathways and biofunctions were ranked by significance as tested by the Fisher's exact test. Canonical pathways were grouped into Signalling Pathways and Metabolic Pathways. Similarly, biofunctions were grouped into Molecular and Cellular Functions and Disease and Disorders. The number of molecules under investigation which are directly implicated in a given biological pathway or process were highlighted by the ration value function included in the IPA software package.

2.3.12 TLR transfection assay

HEK-293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) supplemented with 10% foetal-calf serum for between three and seven passages. For the transfection assay HEK-293 cells were plated in 96-well plates at $8x10^3$ cells per well in serum-free DMEM and transfected after 24 hours (50-80% confluent) using the Gene-juice kit (Novagen, Watford, UK) according to the manufacturer's instructions. Amounts of construct per well were 30ng of human TLRs 2, 4 (co-expressing MD-2), 30ng pCD14, 20ng transfection efficiency control renilla luciferase-reporter construct (pRL-TK) and 10ng firefly luciferase-reporter construct driven by the NF*k*B dependent E-selectin

promoter (pELAM) cloned into pGL3 (Promega). Cells were grown for 2-3 days post-transfection prior to 18 hour challenge. Reporter levels were normalised to co-transfected renilla luciferase and represented as fold induction relative to cells cultured in medium alone ± S.D. Endogenous expression of TLRs in HEK-293 was ruled out by RT-PCR.

2.3.13 Phenol extraction of LOS and LPS

Bacteria were heat inactivated at 65^{o}C for 1 hour. Bacteria were then centrifuged at 6000 rpm for 10 minutes (Sorvil centrifuge, SS34) and the supernatant discarded. The pellet was resuspended at 10g/ml in dH_2O and the cells were blended to a paste on ice using a homogeniser (Tissue Tearor, Biospec products Inc, Bartlesville, USA). An equal volume of 85% liquid phenol (Sigma-Aldrich) was added and the solution was homogenised on ice for 5 minutes. The mix was heated to 65^{o}C and homogenised for 1 minute at 2 minute intervals for a total of 10 minutes. The mix was then centrifuged at 600 x *g* at 4^{o}C for 10 minutes. The aqueous layer was removed and stored temporarily at 4^{o}C. An equal volume of dH_2O was added to the remaining sample and homogenised on ice repeating the steps outlined above. The combined aqueous layers were then dialysed at 4^{o}C for three days in six changes of 5 litres of dH_2O using a 3.5kDa MWCO (molecular weight cut off) dialyser tube (Novagen, D-Tube Dialyzer Maxi). The resulting extract was then stored at -20^{o}C.

2.3.14 Determination of LPS and LOS purity by silver stain

SDS-PAGE electrophoresis of LPS and LOS was carried out using a NuPAGE 4-12% Bis-Tris gel (Invitrogen) according to the manufacturer's instructions. 5µg LPS/LOS was added to 5µl Tricine SDS sample buffer and 1µl Nupage reducing agent (Invitrogen). The sample was heated to 95^{o}C for 5 minutes and 10µl was loaded onto the gel. The gel was run in Tricine SDS buffer (Invitrogen) at 125V for 90 minutes. The gel was then subjected to silver staining using a SilverQuest staining kit (Invitrogen) as per the manufacturer's instructions.

2.4 Protein profile of human serum

2.4.1 Patients and serum sample preparation

Venous blood samples were collected from patients enrolled in the Feiring Heart Biopsy Study after a minimum of four hours fasting. The subset of patients from the Feiring Heart

Biopsy Study examined included 16 patients with RA undergoing CABG (RA+CVD cohort), 19 patients without RA or any other form of IRD undergoing CABG (CVD cohort), 21 patients with RA and but no detectable CVD symptoms and 21 healthy controls (absence of any clinically significant disease, surgery or trauma; absence of drug or alcohol abuse; absence of pregnancy). RA was confirmed according to the accepted ACR-EULAR diagnostic criteria (*table 1.2*). Patient characteristics are outlined in *table 5.1*. Whole blood was collected in sterile 4ml vacutainers without additives and allowed to coagulate at room temperature (<2 hours). The samples were then spun to allow isolation of serum at 2800 x *g* for 20 minutes at 4^{o}C. The serum was removed by pipette and transferred into a sterile 1.5ml tube for storage at -20^{o}C until use.

2.4.2 Luminex assay of human serum

Serum samples from healthy individuals (n = 21), CVD patients (n = 19), RA patients (n = 21) and patients diagnosed with both RA and CVD (n = 16) were analysed using a multi cytokine and chemokine bead array detection system capable of detecting human IL-1β, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 p40/p70, IL-13, IL-15, IL-17, VEGF, TNFα, IFNα, GMCSF, MIP1a, MIP1b, IP-10, MIG, Eotaxin, RANTES, MCP-1, EGF, GCSF, FGF, HGF and IL-2R according to the manufacturer's instructions (Human Cytokine Thirty-Plex Antibody Bead Kit, Invitrogen). Briefly, 25µl 1 x beads (2.5×10^{6} beads ml^{-1} cytokine^{-1}) with defined spectral properties were covalently conjugated to analyte specific monoclonal antibodies and then pipetted into each well of a 96-well filter bottom microplate. The conjugated beads were exposed to 50µl of serum sample or standard solution containing a known concentration of each cytokine and incubated at room temperature on an orbital shaker (500-600 rpm) for two hours while being protected from light exposure. After several washes and filtration by vacuum manifold to remove unbound protein, biotinylated detection antibodies specific for the epitopes of interest were added to the reaction and incubated for 1 hour at room temperature on an orbital shaker (500 – 600 rpm). Following this incubation step, a series of washes and a vacuum filtration to remove unbound antibody was performed. The reaction mixture was then detected by the addition of Streptavidin-R Phycoerythrin (Streptavidin-RPE) which binds with high affinity to the biotinylated antibodies. A further series of washes and vacuum filtrations were then performed to remove any unbound material. The beads were then resuspended in 100µl working wash solution and stored in the dark at 4^{o}C until analysis. The reaction mixtures were analysed using a Luminex flow cytometer (Invitrogen). The

lower detection limit for each mediator measured was 2pg/ml, any values lower than this were considered as 0 for this study. The upper limit of detection was variable depending on the analyte and was dictated by the generated standard curve.

2.4.3 sST2 ELISA

96 well half-area ELISA plates (Corning Costar, Dorset, UK) were coated overnight at room temperature with 100µl/well of sST2 capture antibody (R&D Systems, Abingdon, UK) diluted to 2µg/ml in PBS. Plates were washed twice with PBS/0.1% Tween (Sigma-Aldrich) then blocked for 1 hour at 37^{o}C with 300µl PBS/1% BSA (Sigma-Aldrich) per well. After two washes in PBS/Tween followed by two washes in PBS, sST2 standards were diluted in PBS/BSA and added at 100µl per well to provide a standard curve. A seven point standard curve using two-fold serial dilutions with a high standard of 2000pg/ml was performed. Serum samples were then added at 100µl per well and incubated at room temperature for two hours. Plates were then washed with PBS/Tween before adding 100µl biotinylated detection antibody diluted to a working concentration of 100ng/ml in PBS/0.1% Tween supplemented with 2% heat inactivated normal goat serum. Plates were then incubated for 2 hours at room temperature. Once washed, 100µl of Streptavidin-HRP diluted to a working concentration of 2µg/ml in PBS/0.1% Tween was added to each well for 20 minutes at room temperature. Following another wash step 100µl substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine) (R&D Systems) was added to each well and incubated at room temperature for 20 minutes in the dark. Reactions were then stopped using the appropriate Stop Solution (2N H_2SO_4) (R&D Systems). sST2 concentration was then quantified with reference to the standard curve at 450 nm of a plate reader (Tecan, Sunrise and Magellan, Reading, UK).

2.5 Lipoprotein isolation and oxidative modification

2.5.1 Lipoprotein isolation

50ml venous blood was taken from each of three healthy donors for LDL isolation. All donors underwent a period of 12 hours fasting prior to blood collection. Blood was collected in 0.4 mmol/EDTA. Plasma was isolated from blood by centrifugation at 3000rpm at 10^{o}C for 30 minutes (L-80 ultracentrifuge, Beckman Coulter). LDL isolation

was performed by density adjustment of plasma using potassium bromide (KBr) and discontinuous ultracentrifugation at 50,000 rpm at 10°C (L-80 ultracentrifuge, Ti 70 rotor). On the first day the non-protein solvent density of the pooled serum sample was adjusted to 1.022 g/ml by the addition of 0.2 g dry salt KBr per 10ml of plasma. Following adjustment, the plasma was centrifuged at 10,000 rpm for 18 hours at 10°C. On day two the non-protein solvent density of the serum sample was adjusted to 1.019 g/ml by the addition of solid salt KBr according to the following formula:

$$KBr\ [g] = \frac{volume\ x\ (density\ A - density\ B)}{1 - 0.267\ x\ \frac{density\ A + density\ B}{2}}$$

where volume is the volume of the fluid (ml), density A is the desired density (g/ml), and density B is the actual density. Following adjustment, the plasma was centrifuged at 10,000 rpm for 18 hours at 10°C. The top fraction (VLDL) was carefully removed and discarded. The remaining plasma had the density adjusted to 1,063 g/ml by addition of KBr using the equation above and was again subjected to centrifugation at 10,000 rpm for 18 hours at 10°C. The top layer containing the LDL was gently removed using a pipette. Finally, the LDL stock was dialysed in dialysis tubes with a pore size of 50,000 Daltons (Millipore, Watford, UK) against 5 litres of PBS supplemented with 0.2mM EDTA in the dark (Note: if it was intended to oxidise the LDL stock immediately, there was no need to dialyse in the presence of EDTA). Dialysis was performed over three days with two changes of buffer. The resulting LDL was the stored at 4°C in the dark.

2.5.2 Lipoprotein oxidation

Prior to oxidation, the LDL stock was dialysed in dialysis tubes with a pore size of 50,000 Daltons (Millipore) against 5 litres of PBS to remove all traces of EDTA. Dialysis was performed over three days with two changes of buffer. The preparation was then passed through a 0.45µm filter (Millipore) to sterilise and remove lipoprotein aggregates. 150µl of this preparation was stored at 4°C for a modified Lowry protein determination (*Section 2.5.3*). The remaining LDL stock (2mg/ml) was oxidised by addition of 10µM $CuSO_4$ and incubated for 18 hours at room temperature in the dark. To stop the oxidation reaction and prevent further modification 0.25mM EDTA was added to the solution. Oxidised LDL was again sterilised by passing through a 0.45µm filter and then stored at 4°C in the dark.

2.5.3 Modified Lowry protein assay

The modified Lowry protein assay was used to measure the final concentration of purified LDL. A 2x Lowry concentrate was prepared by mixing three parts copper reagent (*appendix 3*) to one part (1% sodium dodecyl sulphate) and one part (1M NaOH). A standard was prepared from 0.2mg/ml bovine serum albumin (BSA) (Thermo Scientific). Eight two-fold serial dilutions were made of both the LDL and the BSA. 50µl of 2x Lowry solution was added to every 50µl of sample and mixed thoroughly before incubating at room temperature for 10 minutes. After this, 25µl of 0.2N folin reagent (VWR) was then added and incubated at room temperature for 30 minutes. Finally, the samples were analysed using a plate reader at 502nm to obtain OD readings. A standard curve was prepared from the BSA and the concentration of LDL was extrapolated.

2.5.4 Lipoprotein electrophoresis

Lipoproteins isolated from the blood of healthy donors were oxdatively modified by copper sulphate. Electrophoresis was used to confirm that oxidative modification was occurring as necessary. Native LDL from the same samples was used as a control. Prior to agarose gel electrophoresis lipoprotein was stained with Nile Red. This allows LDL to be visualised by UV light following electrophoresis. 20µl of working Nile Red solution (10µg per ml of acetone) (Sigma-Aldrich) was added to 1.5ml tubes and allowed to evaporate fully. 50µl LDL was added to the tube and agitated for 10 seconds, followed by addition of 5µl of 30% w/v sucrose solution. A 0.6% agarose gel in 50mM barbital buffer, pH 8.6 was prepared by microwaving (*appendix 3*). The agarose solution was allowed to cool for five minutes and then poured into the running tray. Once the gel was fully set the electrophoretic chamber was filled with 50mM barbital buffer, pH 8.6. The gel was loaded with 15µl of Nile Red stained LDL and subjected to electrophoresis at 56V for 2 hours. The gel was imaged under UV light (Alpha Innotech, Alphalmager, East Sussex, UK).

2.6 *In vitro* foam cell investigation

2.6.1 Foam cell formation

Human primary macrophages were isolated (*section 2.3.5*) and cultured for six days in 24 well tissue culture plates (BD Biosciences, UK) with RPMI (Life Technologies)

supplemented with 15ng/ml M-CSF. Cells were then incubated with 200µg/ml oxLDL for 1 hour. Foam cell formation was then visualised following Oil-red-O staining (*section 2.6.2*).

2.6.2 Oil-red-O staining and foam cell quantification

Oil-red-O staining was used to image lipid uptake. The steps outlined here were optimised for cells cultured in a 24-well plate. All reagents were used at room temperature. Cell culture medium was removed and the cells were twice washed gently with 250µl PBS. Following complete removal of the PBS, cells were incubated in 250µl 10% formalin/PBS for 15 minutes and then washed twice in ddH_2O. Cells were then washed in 250µl of 60% isopropanol/H_2O for 5 minutes. Following complete removal of the wash solution, cells were incubated with Oil-Red-O working solution (*appendix 3*) for 30 minutes. The cells were then washed in ddH_2O until the water rinsed off clear. The cells were then immersed in 200µl ddH_2O and the intracellular distribution of lipid droplets was imaged by light microscopy (Olympus IX51).

2.6.3 Luminex assay of GM-CSF stimulated macrophages

Human primary macrophages were isolated (*section 2.3.5*) and cultured for six days in 24-well tissue culture plates (BD Biosciences) with RPMI (Life Technologies) supplemented with 15ng/ml M-CSF. Cells were then stimulated overnight with 125ng/ml GM-CSF or vehicle control before the supernatants were removed and subjected to Luminex analysis as described in *section 2.4.2*.

2.6.4 Dextran sulphate-mediated inhibition of foam cell formation

Human primary macrophages were isolated (*section 2.3.5*) and cultured for six days in 48-well tissue culture plates (BD Biosciences) with RPMI (Life Technologies) supplemented with 15ng/ml M-CSF. Cells were then stimulated overnight with 125ng/ml GM-CSF or vehicle control. The following day cells were challenged with 200µg/ml oxLDL and varying concentration of dextran sulphate (Millipore) (0, 2 10, 20 and 40µg/ml) and

incubated at 37^{o}C in 5% CO_2 for 24 hours. Resulting foam cell formation was then visualised following Oil-red-O staining (*section 2.3.9*).

2.7 Quantitative PCR

2.7.1 cDNA synthesis and normalisation

cDNA was synthesised according to the manufacturer's instructions using the supplied reagents (Superscript III First-Strand Synthesis System, Invitrogen) (Note: this method of cDNA synthesis was specific for qPCR, and differed from the method outlined in *section 2.3.8*). 120ng RNA was diluted in 4µl RNase-free H_2O (Qiagen) and 1µl random primers and 1µl dNTPs was added to each sample. Samples were placed in the thermocycler for 5 minutes at 65^{o}C and then on ice for 1 minute. The cDNA synthesis mix was prepared on ice by mixing 2µl of 10x RT buffer, 4µl 25mM $MgCl_2$, 2µl 0.1M DTT, 1µl RNaseOUT and 1µl SuperScript III Reverse Transcriptase. 10µl cDNA Synthesis mix was added to each sample. For the control samples, Superscript III Reverse Transcriptase was replaced by an equivalent volume of RNase-free H_2O. Samples were placed in the thermal cycler for 50 minutes at 50^{o}C then 85^{o}C for 5 minutes. In order to remove any remaining RNA, 1µl RNase-H was added to each cDNA sample and incubated at 37^{o}C for 20 minutes. cDNA was stored at -20°C.

2.7.2 Primer design

Sets of primers were designed for quantitative PCR using the PrimerBLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primer sets used are described in *appendix 4*.

QPCR primer specifications were as follows:

- Amplicon size less than 150bp
- Between 18 and 23 base pairs in length
- Between 40 and 65% GC content (50% optimal)
- Maximum 3' self complimentarity: 1
- Maximum self complimentarity: 2
- Tm of primers within 59.5^{o}C and 61^{o}C (60^{o}C optimal)

- Not more than two G or C bases in the last five bases at 3' end of each primer (i.e. no GC clamp)
- Avoid stretches of four or more G or C bases in a row

If these conditions did not support generation of appropriate primers then the self complimentarity was relaxed from two to three and the primer Tm reduced to 59^{o}C. The only condition that was never relaxed was maximum 3' self complimentarity.

BLAST analysis was used to ensure that primer sequences were specific to the gene of interest alone and that no splice variants existed that could confuse the analysis.

2.7.3 SYBR QPCR protocol

Each QPCR reaction was performed in triplicate. In order to ensure homogeneity a master mix was prepared for each reaction prior to plating out triplicates. Each master mix reaction contained:

- 33µl SYBR Green FastMix (Quanta Biosciences, Lutterworth, UK)
- 23.3µl neuclease free water (Qiagen)
- 1.2µl primer mix (each primer mixed together at 50pmol/µl)
- 4µl cDNA (equivalent to 24ng cDNA)

This was then mixed by pipetting at least five times. 20µl of the master mix was then pipetted into each of the three wells (triplicates) on a 96-well QPCR plate (Star Lab, Milton Keynes, UK). A non-template control (NTC) was included to determine the level of contamination and to show the detection limit of the assay. The plate was spun for 10 seconds at 400 x *g* to ensure that the contents of each well were at the bottom of the plate. Using the 9700HT QPCR machine (Applied Biosystems) a fast run was performed, which used the following reaction conditions:

- Initial three minutes at 94^{o}C (not repeated)
- 5 seconds at 94^{o}C then 15 seconds at 60^{o}C, repeated 40 times (measuring fluorescence at the end of each 60^{o}C elongation step).

2.7.4 Analysis of QPCR data

Gene expression was quantified using the relative quantification (RQ) method. This technique utilises the formula $2^{-\Delta\Delta CT}$ in order to calculate the relative number of gene transcripts. The C_T value represents the PCR cycle at which the amplified gene target reaches a defined threshold. 40 PCR cycles were performed; accordingly the C_T values can range from 0 to 40. C_T values for each triplicate were measured and the mean calculated. If a C_T value was greater than 34, the data was dismissed. C_T values were calculated for the stimulated samples and the non-stimulated control samples. The C_T of the endogenous control gene 18S was also calculated. The ΔC_T value was calculated [ΔC_T = C_T (target gene) − C_T(18S)]. This was performed for each gene in both the stimulated sample and the non-stimulated control sample. The relative expression of each gene was then calculated with the formula $\Delta\Delta C_T = \Delta C_T$ (positive sample) − ΔC_T(control sample). This was then expressed in terms of fold change relative to the control sample with the formula [fold change = $2^{-\Delta\Delta CT}$]. For down-regulated genes (value less than 1) the negative inverse of the value was taken. This allowed down-regulation to be expressed as a negative value.

2.8 FACS analysis of scavenger receptors

Human primary macrophages were stained independently for both MARCO (extracellular) (Santa Cruz, Middlesex, UK) and SR-B1 (intracellular) (Novus Biologicals, Cambridge, UK). Macrophages were washed in PBS and then resuspended in 50µl FACS buffer. Cells were then incubated with 5µl Fc Block (BD Biosciences) for 15 minutes at 4^{o}C before being spun down at 400 x *g* for five minutes. For antibodies specific to extracellular proteins (MARCO) cells were then aspirated and then incubated with 1µl un-conjugated rabbit anti-human primary antibody specific to MARCO (Santa Cruz) or rabbit IgG as the isotype control (BD Biosciences) for 15 minutes at 4^{o}C in the dark. For antibodies specific to intracellular proteins (SR-B1), macrophages were first permeabilised by incubating in 250µl cytofix/cytoperm (BD Biosciences) for 15 minutes at 4^{o}C in the dark before being washed twice in 1x permwash (BD Biosciences). Cells were then aspirated and then incubated with 1µl of an un-conjugated rabbit anti-human primary antibody specific to SR-B1 (Southern Biotech, USA) or rabbit IgG as the isotype control (BD Biosciences) for 15 minutes at 4^{o}C in the dark. For both extracellular and intracellular specific antibodies, cells were then resuspended in 1ml FACS buffer or permwash respectively, before being

spun at 400 x *g* for 5 minutes and aspirated. Samples were then incubated with anti-rabbit IgG-PE (Southern Biotech, Cambridge, UK) for 15 minutes at 4^{o}C in the dark. Finally the stained cells were spun down at 400 x *g* for five minutes before being resuspended in 350µl FACS buffer and acquired on a MACSQuant (Miltenyi Biotec, Surrey, UK). The resulting data was analysed using Flowjo software (Treestar).

3 Cellular composition and cytokine expression in the aortic adventitia of human subjects with cardiovascular disease in the presence and absence of rheumatoid arthritis

3.1 Introduction

RA is a chronic systemic inflammatory disorder that principally affects synovial joints. RA is associated with increased CVD as a result of accelerated atherosclerosis. Although the underlying mechanisms of vascular comorbidity in RA are not known, it is hypothesised that the increased risk of CVD is driven by systemic inflammation and/or increased expression of autoantigens (Sattar and McInnes, 2005).

The extent of systemic inflammation is significantly greater in RA patients than in individuals without the disease. Indeed, inflammation in RA patients is often well above the levels that arise as a result of lifestyle factors, including smoking, obesity, reduced activity or as a result of blood vessel pathology. Several studies support the hypothesis that elevated inflammation plays a fundamental role in the accelerated CVD observed in RA:

- Murine studies demonstrate that pro- inflammatory cytokines promote atherogenesis, whereas inhibition of these cytokines reduces CVD risk (Huber et al., 1999, Steffens et al., 2005)
- Therapeutic reduction of inflammation in RA improves endothelial function and lipid profile (Dixon and Symmons, 2007)
- RA severity predicts CVD risk (Jacobsson et al., 2001, Maradit-Kremers et al., 2005)
- CVD risk in RA cannot be accounted for by traditional risk factors (del Rincon et al., 2005).

Analysis of protein and mRNA has revealed that many pro-inflammatory cytokines such as IL-1, IL-6, TNFα, GMCSF and TGFβ are abundant in the synovium and serum of patients with RA (Feldmann et al., 1996). The release of pro-inflammatory cytokines in RA may be crucial in the initiation of co-morbid CVD. It has been demonstrated that TNFα is capable of initiating a pro-inflammatory response and acts as an alarm to injury. If the release of TNFα is blocked, expression of pro-inflammatory cytokines such as IL-6 and IL-1 will decrease (Fong et al., 1989). TNFα-mediated induction of numerous cytokines, chemokines and adhesion molecules (Doukas and Pober, 1990, Schroder et al., 1990) is of key importance in attracting leukocytes to areas of injury. TNFα is a pleiotropic cytokine that orchestrates systemic inflammation and stimulates the acute phase response. TNFα has previously been detected in both the RA synovium (Ohta et al., 2001) and murine atherosclerotic lesions (Zhang et al., 2007). The reduction of inflammation in RA that is

observed following anti-TNFα therapy appears to be associated with a concomitant reduction in CVD risk (Avouac and Allanore, 2008). As RA patients have elevated levels of systemic TNFα, we hypothesise that TNFα may be over-expressed in the aortic tissue of these individuals, which may be associated with an increased risk of CVD.

Atherosclerosis is a chronic inflammatory disease in which both humoral and cell-mediated immune responses participate (Hansson, 1997). In advanced stages of atherosclerosis, inflammatory cell infiltrates are often detected in the aortic adventitia adjacent to sites of atherosclerotic plaque. Immunohistochemical studies by a previous PhD student (Ahmed, 2010) in our group showed small aggregates of $CD20^{+}$ B cells and occasional plasma cells in the aortic adventitia of CVD patients (unpublished results). These observations suggest that lymphoid follicle-like organisations, which develop in a variety of autoimmune mediated inflammatory diseases such as RA (Randen et al., 1995), may be present in the aortic adventitia of CVD patients.

Follicular dendritic cells (FDCs) form a contiguous network that is vital for formation and survival of germinal centres in response to signals that include TNFα. Mice expressing a mutant form of TNFα that cannot be released do not form primary follicles (Ruuls, 2001). In primary follicles, FDCs are localised in the central region, whereas germinal centre FDCs exhibit a polarised distribution. In both primary follicles and germinal centers, FDCs and mature B cells are recognised by antibodies to CD21, also known as complement receptor 2 (Imal and Yamakawa, 1996). CD21 is required for germinal centre development, antibody production and B cell tolerance. To evaluate whether lymphoid follicle-like structures were present in patients with CVD, we used immunohistochemical staining for CD21.

The role of autoantigens in the onset of atherosclerosis and RA has gained support in recent years. Heat shock proteins (HSPs) are highly conserved molecules that execute a range of functions including assembly, folding and translocation of proteins (Hightower, 1991). In addition to being molecular chaperones, HSPs are immunodominant molecules and a substantial proportion of the immune response against pathogenic microorganisms is targeted towards pathogen-derived HSP peptides (Kaufmann, 1990, Young, 1990). Given the phylogenetic homology between prokaryotic and mammalian homologues of these molecules (for example, 60% of residues are shared across the HSP60 family) there is a concept that pathogen-derived HSPs may be recognised by the host immune system as autoantigens, in a process known as molecular mimicry (Kaufmann, 1990).

Immunological recognition of cross-reactive HSP epitopes could present a link between autoimmunity and infection (Lamb et al., 1989).

Among the immuno-pathological factors shared by RA and atherosclerosis, a subset of $CD4^+/CD28^-$ T cells have been implicated. HSP60 is a mitochondrial chaperone protein responsible for ATP dependent folding of 15-30% of total cellular protein. It has been identified as a dominant autoantigen recognised by $CD4^+/CD28^-$ T cells (Zal et al., 2004). As previously highlighted, molecular mimicry between host and pathogen HSPs may lead to the targeting of self HSP60 by auto- reactive T and B cells. Although HSP60 is normally only expressed intracellularly, it can also be expressed on the surface of endothelial cells in response to pro-inflammatory cytokines such as TNFα and IL-1α (Xu et al., 1994). We hypothesised that the systemic proinflammatory profile of RA patients may lead to overexpression of HSP60 in aortic tissue, triggering a $CD4^+/CD28^-$ T cell autoimmune response that leads to an increased risk of atherosclerosis.

HSP47 is a heat shock protein and molecular chaperone that interacts with procollagen during its folding, assembly and transport from the endoplasmic reticulum in mammalian cells (Tasab et al., 2000). Histological studies demonstrate that little or no HSP47 is expressed in healthy artery samples. Conversely, HSP47 is observed in ~65% of atherosclerotic tissue and is mostly expressed in lipid-rich regions of the fibrous cap and smooth-muscle-like cells (Rocnik et al., 2000). Collagen plays a pivotal role in the pathogenesis of both CVD and RA. The accumulation of fibrillar collagen during plaque formation can trigger arterial stenosis. Conversely, inadequate collagen production or failure in the breakdown of collagen can lead to plaque rupture (Davies and Thomas, 1984). RA is categorised as a collagen vascular disease — a group of diseases that also includes dermatomyositis and scleroderma — which affects the collagen that constitutes connective tissue, bones and tendons being affected (Das, 1991). In RA, an autoimmune reaction is mounted against collagen structures and ultimately leads to their destruction (Kim et al., 2004). As HSP47 is necessary for correct and regulated collagen expression, we hypothesised that dysregulation of HSP47 may be central to pathogenesis of both CVD and RA. Furthermore, HSP47 antigen and antibody levels have been demonstrated to be significantly elevated in patients with RA (Yokota et al., 2003).

Recently, two novel pro-inflammatory proteins belonging to the S100 family of calcium-binding proteins have been characterised: S100A8 and S100A9 (Striz and Trebichavsky,

2004). These two proteins can form either monovalent homodimers or a heterodimer, termed calprotectin, in a calcium-dependent manner (Mortensen et al., 2009). Calprotectin is principally expressed in neutrophil granulocytes, however the level of expression is dependent upon inflammatory status and stage of myeloid cell differentiation (Striz and Trebichavsky, 2004). Current knowledge of the expression of calprotectin on other cell populations is limited. Research into the regulation of calprotectin has elucidated that calprotectin expression can be induced by a variety of stimuli, including bacterial LPS, IFN-γ and TNF-α (Xu and Geczy, 2000). The principal function of calprotectin is as an antimicrobial agent. However, calprotectin can also participate in various innate immune processes, including chemotaxis and cellular adhesion (Ryckman et al., 2003). We hypothesise that calprotectin expression will be detectable in the aortic adventitia of CVD patients and that calprotectin expression would be dysregulated in subjects with systemic inflammation resulting from RA. Furthermore, detection of calprotectin in the aortic adventitia may indicate previous or present exposure to potentially pathogenic bacteria.

To increase understanding of the link between RA and CVD, we collected aortic adventitia samples during CABG surgery from 20 patients with RA and 19 control patients matched for age, family history of CVD, BMI and other traditional CVD risk factors.

The objective of the research described in this chapter was to determine and compare the expression of several possibly pro-atherogenic proteins and to determine the cellular composition in the aortic adventitia of patients with CVD either with or without RA. The principal aims of this study were:

1. Quantify the extent of inflammatory infiltration within the aortic adventitia of RA patients with CVD compared to patients with CVD alone.

2. Determine whether the aortic adventitia of RA patients with CVD has an association with TNFα, which may orchestrate an elevated pro-atherogenic response, and to relate expression of TNFα to presence of inflammatory infiltrate.

3. Investigate the presence of CD21, a marker of follicular dendritic cells, to determine whether lymphoid follicle-like organisations exist in the aortic adventitia of CVD patients.

4. Determine if the potential autoantigens HSP60 and HSP47 are present within the aortic adventitia of RA patients with CVD in comparison to CVD patients without RA .

5. Detect and quantify calprotectin, a pro-inflammatory and anti-microbial protein, in the adventitia of RA patients with CVD and compare to patients with CVD alone.

3.2 Results

3.2.1 Patient characteristics

Human aortic adventitia biopsy samples were from the Feiring Heart Biopsy study. All biopsies were obtained from patients undergoing routine coronary artery bypass graft (CABG) surgery. The demographic data and prevalence of traditional CVD risk factors was similar in the group with RA and the control group (CVD only) (*table 3.1*). The time period from initial angiography to CABG surgery was shorter for RA patients than control patients. RA patients had higher levels of inflammatory biomarkers. RA patients were treated with a range of steroidal and non-steroidal anti-inflammatory drugs. A total of 39 sections were included in the following studies, which comprised patients with RA and CVD (n=19) and patients with CVD only (n=20).

3.2.2 Morphology of aortic adventitia sections

Aortic adventitia sections were stained with Haematoxylin & Eosin and examined by light microscopy. The aortic adventitia biopsies predominantly consisted of smooth muscle cells and connective tissue fibres and contained variable amounts of adipose tissue. Small vessels, termed *vasa vasorum* were regularly visible; these vessels function to supply the adventitia with oxygen and essential nutrients. Endothelial cells surrounded the *vasa vasorum*. Nerves (*nervi vasorum*) were detectable in several sections. Adventitial inflammatory infiltrates were frequently observed, consistent with biopsies being from patients with CVD. The aortic adventitia was contained within a mesothelial layer. *Figure 3.1* illustrates these key cell types.

	RA (n=19)	Non-RA (n=20)	*P* value
Age – years	69±9	68±9	0.746
Males – no. (%)	12 (63)	14 (70)	0.651
Duration of CVD – years	6.3±8.7	7.2±6.6	0.378
History of myocardial infarction – no. (%)	15 (79)	9 (45)	0.048
Acute coronary syndrome – no. (%)	9 (47)	4 (20)	0.096
Time from angiography to CABG – days	17±30	31±60	0.944
Left ventricle ejection fraction – no. (%)	59±13	64±13	0.237
C-reactive protein – mg/litre	23±40	3±3	0.006
Erythrocyte sedimentation rate – mm/hour	34±29	15±10	0.047
Serum PTX3	2.07±1.00	1.48±0.86	0.067
Body mass index – kg/m^2	25±5	25±2	0.761
Hypertension – no. (%)	13 (68)	9 (45)	0.200
Family history of CVD – no. (%)	11 (58)	20 (100)	0.001
Hyperlipidemia – no. (%)	16 (84)	20 (100)	0.106
Diabetes – no. (%)	2 (11)	1 (5)	0.605
Previous smoker – no. (%)	10 (52)	12 (60)	0.751
Current smoker – no. (%)	2 (11)	1 (5)	0.605
Current use of:			
Oral glucocorticosteroids – no. (%)	8 (42)	0 (0)	0.001
Disease modifying drugs – no. (%)#	16 (84)	0 (0)	<0.001
Cox2-selective inhibitors – no. (%)	5 (26)	0 (0)	0.020
Traditional NSAIDs – no. (%)	2 (11)	0 (0)	0.231
Lipid-lowering drugs – no. (%)	16 (84)	19 (95)	0.342
Acetylsalicylic acid – no. (%)	17 (90)	17 (85)	1.000
Betablockers – no (%)	15 (88)	15 (79)	0.662
ACE inhibitors – no. (%)	5 (31)	6 (30)	1.000
Duration of IRD – years	17 (15)	-	-
Patient global assessment of RA (VAS, 0-100 millimetre)	29±24	-	-
Number of swollen joints	4.2±5.3	0	<0.001
History of aortic aneurysm	1 (5)	0 (0)	0.487

Table 3.1. Patient characteristics

**Unless indicated otherwise, values represent mean ± SD. Numbers may not add up to the expected total due to missing data for some variables. ACE: angiotensin–converting enzyme. CVD: cardiovascular disease. NSAIDs: non-steroidal anti-inflammatory drugs. VAS: visual analogue scale.*

 # Azathioprine=1, suphasalazine monotherapy=2, auranofine=1, CPH-82=1, leflunomide=2, methotrexate monotherapy=7, etanercept and methotrexate=1, suphasalazine and hydroxychloroquine=1.

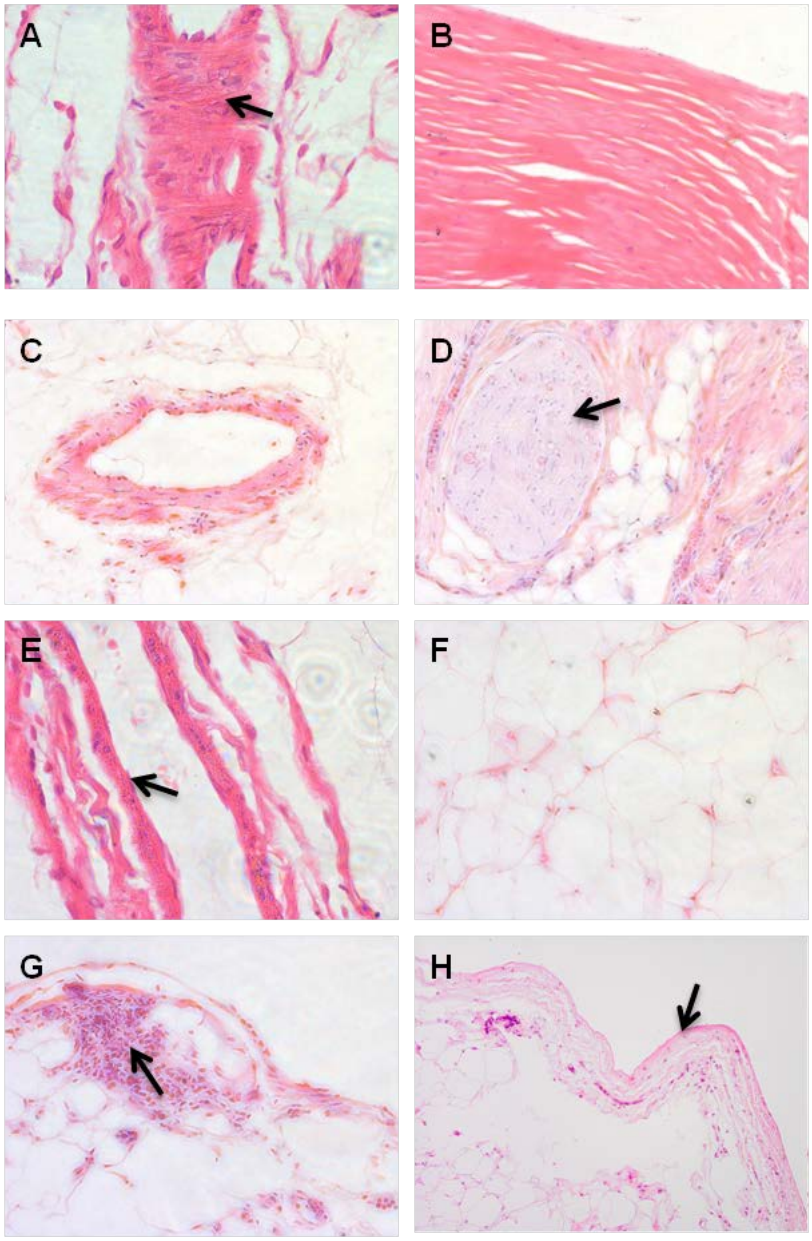


Figure 3.1. Morphological and cellular characterisation of the aortic adventitia

Representation of typical structures observed in the aortic adventitia. Sections were stained with H&E and imaged by light microscopy (200x, unless otherwise stated). Smooth muscle cells (A); Connective tissue fibres (B); Vasa vasorum (C); Nerve (D); Endothelial cells (E); Adipose tissue (F); Mononuclear cell infiltrate (G) and Mesothelial cells (100x)* (H). Arrows have been inserted for clarity.*

**Endothelial and mesothelial cells were distinguished by location. Endothelial cells lined the inner layer of the vasa vasorum. Mesothelial cells form the simple squamous epithelium that lines areas of the adventitia.*

3.2.3 Inflammation in the aortic adventitia

Inflammatory cell infiltrates have previously been detected in the aortic adventitia of patients with CVD (Ramshaw and Parums, 1990). As RA and CVD are both inflammatory conditions, we hypothesised that co-morbid patients may exhibit exaggerated aortic inflammation.

We examined 19 CVD+RA and 20 CVD only aortic adventitia biopsies taken from the Feiring Heart Biopsy Study. Atherosclerotic lesions (plaques or fatty streaks) were detected in 4 of 19 (21%) patients with RA and in 3 of 20 (15%) patients without RA (P=0.63). All lesions were confined to the media of the biopsies (data based on surgeons perioperative observation).

Nine of the 19 patients with RA (47%) and 9 of 20 CVD only patients (45%) had inflammatory infiltrate in the aortic adventitia (*p=0.30*). There was no significant difference between the two cohorts in either the number of inflammatory infiltrates or the size of the largest inflammatory infiltrate per section (*table 3.2*). Inflammatory infiltrates were detected in the perivascular region (around the vasa vasorum), diffusely in connective and adipose tissue and most commonly as a submesothelial infiltrate (*figure 3.2*).

The occurrence of inflammatory infiltrates in the adventitia of 30 matched internal mammary samples was significantly lower than in the corresponding aortic adventitia (0.02%±0.001 and 0.2±0.67 respectively) (*figure 3.5*). The largest inflammatory infiltrate as a percentage of the total section in the aortic adventitia and internal mammary was 3.6% and 0.2% respectively.

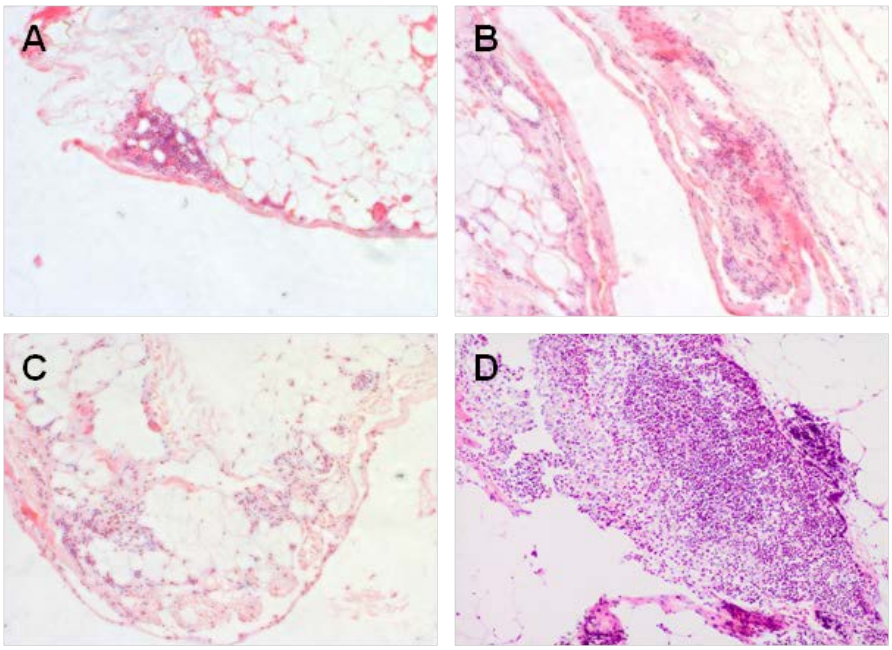


Figure 3.2 Histological examination of aortic adventitia inflammatory infiltration by light microscopy

A, *Localised submesothelial mononuclear cell infiltration in a patient with CVD only.* ***B,*** *Submesothelial mononuclear cell infiltration in a patient with co-morbid RA and CVD.* ***C,*** *Diffuse submesothelial infiltration in a patient with CVD only.* ***D****, Pronounced mononuclear cell infiltration in the inner adventitia in a co-morbid patient. Sections were stained with Haematoxylin and Eosin and imaged with a 10x objective.*

	RA + CVD	CVD	*P* value
No. of adventitial inflammatory infiltrates			*0.22*
0	10 (21)	11 (55)	
1	3 (16)	5 (25)	
2	1 (5)	1 (5)	
3	1 (5)	2 (10)	
>3	4 (26)	1 (5)	
Size of largest adventitial infiltrate as % of section			*0.35*
0	10 (53)	11 (55)	
<0.5	8 (42)	7 (35)	
0.5-1	1 (5)	1 (10)	
1-3	0 (0)	0 (0)	
3-5	0 (0)	1 (10)	
>5	0 (0)	0 (0)	
Total infiltrate as % of section	2.5	3.2	*0.53*

Table 3.2 Number and size of inflammatory infiltrates in the aortic adventitia of patients with RA + CVD and CVD only

Values represent the number (%) of patients. Data represents 19 RA + CVD patients and 20 CVD only patients. Statistical analysis was performed by Mann Whitney U test suitable for non-parametric data.

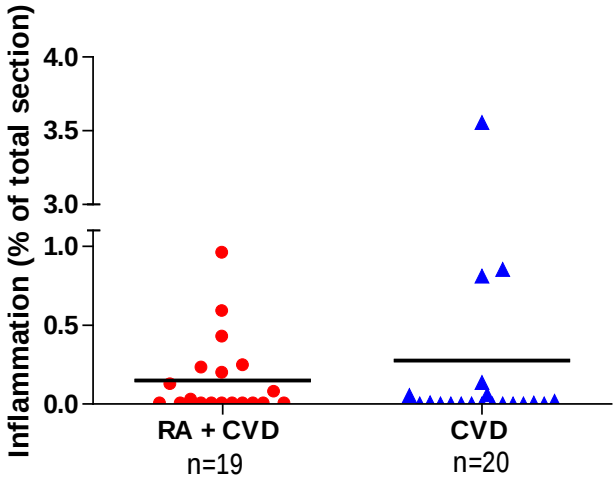


Figure 3.3 Inflammatory infiltrates in the aortic adventitia of RA+CVD and CVD only patients

Sites of inflammation were measured and expressed as a percentage of the total section area. The comparison between the CVD+RA and CVD only group was not significantly different (mean ± SD of 0.15±0.26 and 0.28±0.81 respectively). Statistical analysis was performed with a Mann Whitney U test for non-parametric data. A p-value of <0.05 was considered statistically significant.

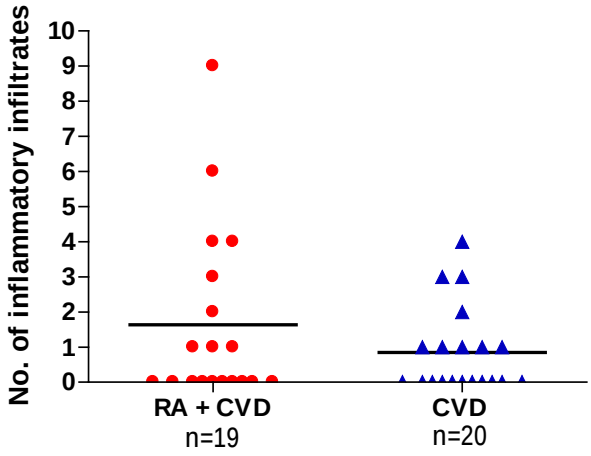


Figure 3.4 Number of inflammatory infiltrates in the aortic adventitia of RA+CVD and CVD only patients

The number of independent inflammatory sites was not significantly different between the RA+CVD group and the CVD only group (mean ± SD of 1.6±2.5 and 0.8±1.2 respectively). Statistical analysis was performed with a Mann Whitney U test for non-parametric data. A p-value of <0.05 was considered statistically significant.

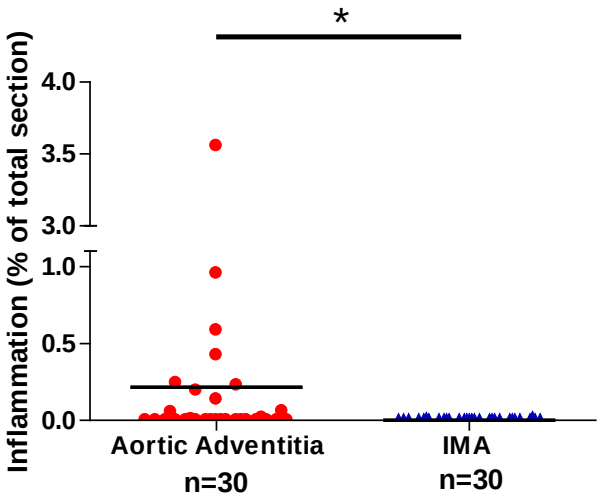


Figure 3.5 ***Aortic inflammation in matched human aortic adventitia and internal mammary artery***

Sites of mononuclear cell inflammation were measured and expressed as a percentage of the total section area. Statistical analysis was performed with a Mann Whitney U test for non-parametric data. A p-value of <0.05 was considered statistically significant and is represented as an (*).

3.2.4 Relationship between vascular inflammation and traditional CVD risk factors

We analysed the relationship between the occurrence of inflammation, the number of inflammatory infiltrates and the total area of inflammatory infiltration in the aortic adventitia with the following factors: sex, age, duration of CVD, BMI, and CRP level. Vascular inflammation did not correlate with any of the CVD risk factors considered here (*table 3.3 and figure 3.6*).

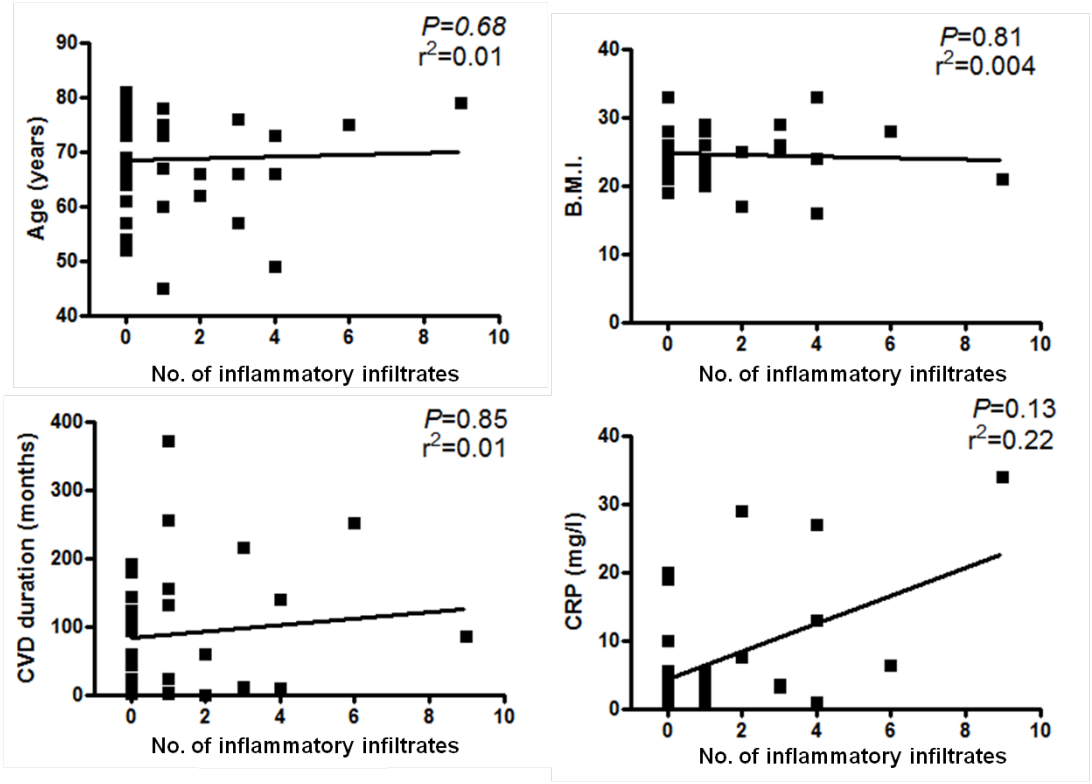


Figure 3.6 Analysis of potential inflammatory infiltrate risk factors

The number of independent inflammatory infiltrates were determined in all patients (n=39) and correlated with a variety of potential risk factors. Representative graphs show correlation of inflammatory infiltrate number with age, B.M.I, CVD duration and CRP. P value was calculated by spearman rho and r^2 by linear regression.

	All Patients (n=39)			CVD only (n=20)			RA + CVD (n=19)		
Predictor	Inflammatory infiltrate presence	Number of infiltrates	Area of infiltrates	Inflammatory infiltrate presence	Number of infiltrates	Area of infiltrates	Inflammatory infiltrate presence	Number of infiltrates	Area of infiltrates
Age	0.64 (ns)	0.68 (ns)	0.80 (ns)	0.52 (ns)	0.44 (ns)	0.79 (ns)	0.94 (ns)	0.88 (ns)	0.58 (ns)
B.M.I	0.70 (ns)	0.81 (ns)	0.65 (ns)	0.81 (ns)	0.68 (ns)	0.81 (ns)	0.54 (ns)	0.62 (ns)	0.81 (ns)
CVD duration	0.85 (ns)	0.85 (ns)	0.55 (ns)	0.80 (ns)	0.87 (ns)	0.70 (ns)	0.52 (ns)	0.59 (ns)	0.12 (ns)
C.R.P	0.66 (ns)	0.13 (ns)	0.70 (ns)	0.11 (ns)	0.12 (ns)	0.41 (ns)	0.43 (ns)	0.99 (ns)	0.45 (ns)
Hypertension	0.43 (ns)	0.75 (ns)	0.67 (ns)	0.31 (ns)	0.36 (ns)	0.27 (ns)	0.43 (ns)	0.46 (ns)	0.67 (ns)
E.S.R	0.21 (ns)	0.57 (ns)	0.48 (ns)	0.82 (ns)	0.67 (ns)	0.38 (ns)	0.56 (ns)	0.05 (ns)	0.39 (ns)

Table 3.3 Predictors of the presence, number and area of inflammatory infiltrates in the aortic adventitia

*Area of inflammatory infiltrates was recorded as a % of the total section area occupied by inflammatory infiltrates. Spearman's rank correlation coefficient (non-parametric) was used to examine statistical dependence. Non-significant (ns); *p<0.05; **p<0.01; ***p<0.001*

3.2.5 Detection of TNFα in the aortic adventitia of CVD patients with and without RA

Having confirmed the presence of inflammatory infiltrates in the aortic adventitia, the predominant inflammation in the vessel made TNFα an obvious choice for investigation. To investigate the presence of TNFα we first had to establish a technique for detection. The initial experiments were carried out on sections of tonsil biopsies, as the number of aortic adventitia sections was limited. To determine the optimal antigen-retrieval method for TNFα immunohistochemistry, a series of methods with varying primary antibody concentration were performed (*figure 3.7*).

3.2.6 TNFα immunohistochemistry established in human tonsil

Tonsil biopsies were fixed in paraformaldehyde and then paraffin embedded. Sections were cut and mounted on glass slides. In order to determine optimal staining conditions, sections were subjected to different antigen retrieval mechanisms and different dilutions of anti-TNFα primary antibody, and the quality of staining was determined by light microscopy. A trypsin retrieval system was used first: this enzymatic reaction is designed to break protein cross-links formed during fixation and unmask antigens and epitopes of interest. The trypsin retrieval technique was desirable as, unlike alternative techniques, trypsin retrieval does not involve a boiling step and leads to less tissue damage during processing. A 1/50 (as per manufacturers recommendation) and a 1/25 dilution of the primary antibody was used. The results demonstrated that this antigen retrieval method was not successful at unmasking the TNFα epitope and rendered this technique unsuitable (*figure 3.7.A and 3.7.B*).

The next antigen retrieval method evaluated used citric acid (pH 6). Due to the boiling stage, this method is considered harsh and could lead to tissue damage. This technique led to clear cell specific TNFα staining and left the isotype control completely clear (*figure 3.7 C and 3.7 D.*). Furthermore, there was no evidence of tissue remodelling or damage following the boiling stage. It was also found that a 1/25 primary antibody dilution gave superior staining to a 1/50 dilution. Additionally, a further improvement to the staining procedure was made by performing a peroxidise block following incubation with the primary antibody, instead of prior.

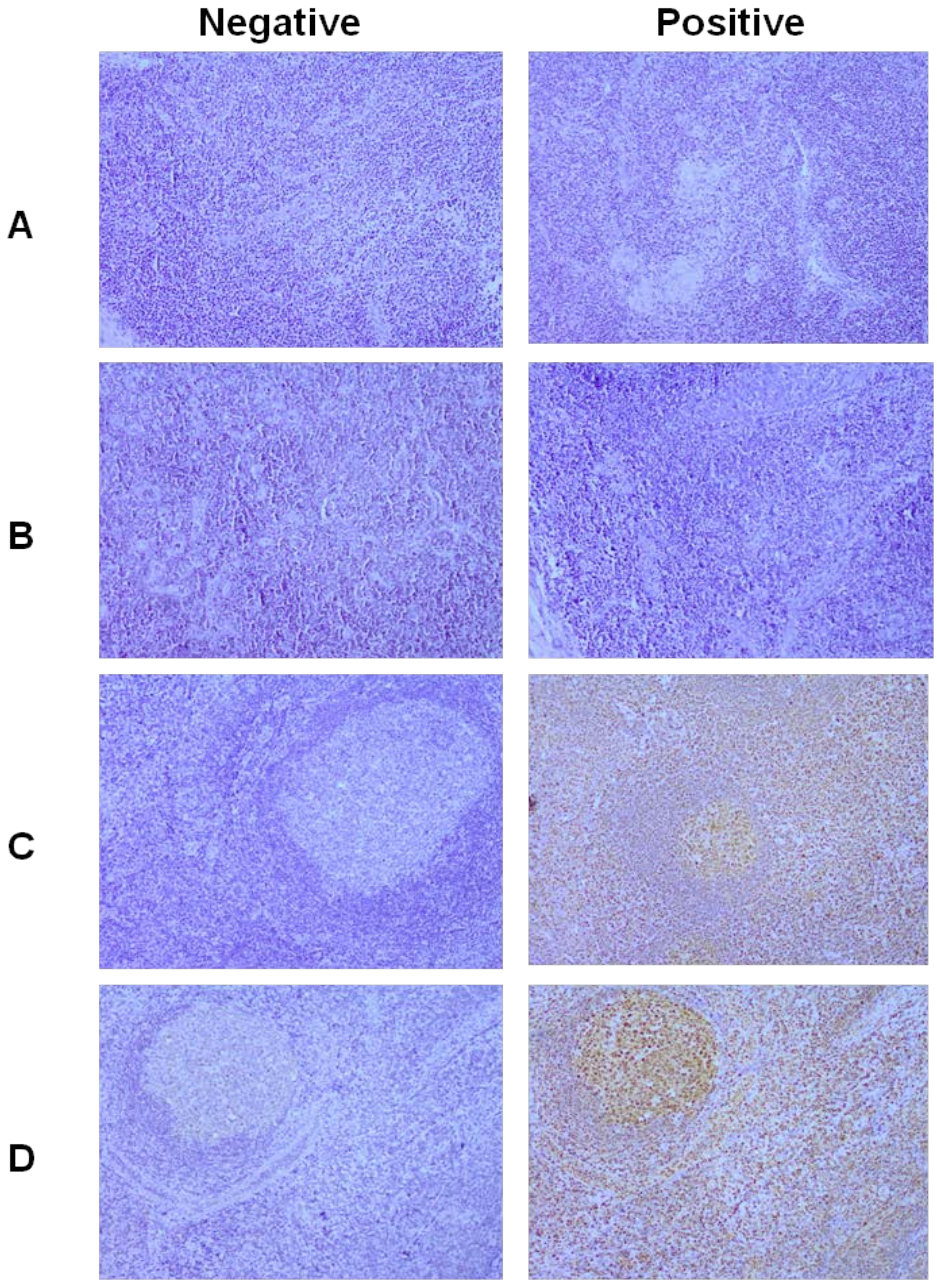


Figure 3.7 TNFα staining using different antibody concentration and antigen retrieval systems

To establish optimal TNFα staining different antigen retrieval systems were tested on tonsil sections. Figures A and B show trypsin retrieval using a 1/50 and 1/25 antibody dilution respectively. Figures C and D show citric acid retrieval using a 1/50 and 1/25 antibody dilution respectively. Figure D shows strong TNFα staining (brown colour) with no background staining detectable in the isotype control. Sections were counterstained with haematoxylin to define nuclei and cytoplasm. Images were acquired by light microscopy with a 10x objective lens.

To summarise, citric acid retrieval with a primary antibody dilution of 1/25 and peroxidise block following primary antibody incubation resulted in clear isotype controls and a strong, cell specific positive staining. Accordingly, this optimised staining technique was used to stain the aortic adventitia sections.

3.2.7 Immunoreactivity for TNFα in RA+CVD and CVD only patients

Tissue was obtained from the aortic adventitia of 19 patients with RA and CVD and 20 patients with CVD only. Mononuclear cell infiltrates expressing TNFα were identified in tissues from both cohorts, with strongest staining in areas of large submesothelial infiltration. TNFα was also detected in inner adventitia inflammatory infiltrates but at a lower frequency. Other cells that displayed immunoreactivity for TNFα included smooth muscle cells that surrounded larger *vasa vasorums*, however this staining was far weaker than that observed in the inflammatory infiltrates. Observable levels of TNFα were present in 12 of the 19 RA+CVD patients and 6 of the 20 CVD only patients (*figure 3.8*).

TNFα staining was scored in a blinded manner. Each section was imaged and the number of independent sites of TNFα staining were recorded. All inflammatory and smooth muscle cells were then imaged at high magnification, and the overall percentage of cells that expressed TNFα was recorded and assigned a score of 0-3 (no staining, >0%<10%, 10-25% and >25% respectively). The TNFα score was significantly greater in the RA+CVD group compared to the CVD only group (p=0.02) (*figure 3.9*). Furthermore, there was a trend towards an increased number of independent TNFα-expressing sites in the RA+CVD group compared to the non-RA group (*figure 3.8*), however the difference did not reach statistical significance (p=0.06).

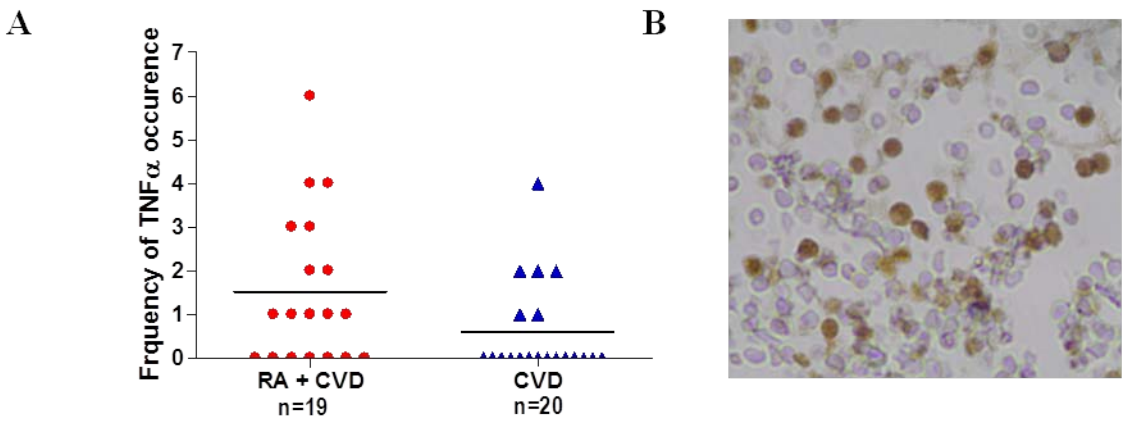
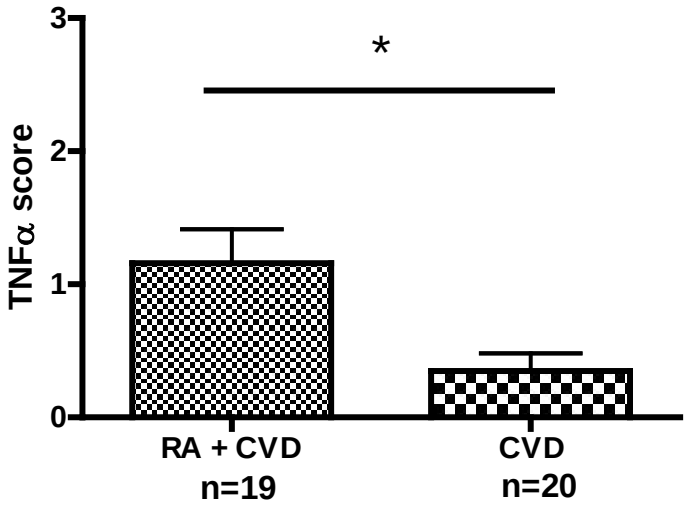


Figure 3.8 Number of sites of TNFα staining in the aortic adventitia of RA+CVD and CVD only patients

(A) The number of independent sites of TNFα staining was not significantly different between the RA+CVD and CVD only group with a mean ± SD of 1.5±1.7 and 0.6±1.1 respectively. Statistical analysis was performed with a Mann Whitney U test for non-parametric data (p=0.06). (B) Representative image of TNFα in an inflammatory infiltrate of the aortic adventitia (400x magnification).



TNFα score (0-3)	RA +CVD (n=19)	CVD only (n=20)	*P* value
0	7	14	*0.02*
1	5	5	
2	4	1	
3	3	0	

Figure 3.9 Quantitative scoring of TNFα in smooth muscle cells and inflammatory cells in the aortic adventitia of RA+CVD and CVD only patients

The number of smooth muscle cells and inflammatory cells were approximately counted and each section was scored according to the percentage of cells expressing TNFα. 0, negative; 1, >0%<10%; 2, 10-25%; 3, >25%. A significant difference was observed between the RA+CVD and CVD only cohort with a mean ± SEM of 1.2±0.26 and 0.3±0.13 respectively. p=0.02 represented (*) (Mann Whitney U test).

3.2.8 Relationship between TNFα and adventitial inflammation

Our data have so far demonstrated that TNFα is expressed in the aortic adventitia of patients with CVD. Furthermore we have observed a significant increase in TNFα expression in patients with co-morbid RA. We also found inflammatory cells are present in the aortic adventitia of a large proportion of patients with CVD (n=39) and that TNFα was principally expressed by inflammatory cells in the tissue. We therefore hypothesised that, in the aortic adventitia, TNFα expression would positively correlate with the number of inflammatory infiltrates. A linear regression analysis revealed that the number of sites of TNFα expression did not significantly correlate with the number of inflammatory infiltrates when all patients were included (n=39) (p=0.055, r^2=0.26), however there was a strongly significant correlation when considering only the RA+CVD patients (p=0.0058 **, r^2=0.59) (*figure 3.10 & figure 3.11*). These data suggest that the increased CVD burden in RA patients may be partly explained by an increase in risk of TNFα expression within inflammatory infiltrates of the aortic tissue.

3.2.9 Relationship between TNFα expression and traditional CVD risk factors

The relationship between the number of independent TNFα sites and the extent of TNFα staining (TNFα score) in the aortic adventitia with the following factors was analysed: age, duration of CVD, BMI, ESR, hypertension and CRP level. When considering all patients (n=39), CRP positively correlated TNFα score (p=0.02). However significance was lost when considering RA+CVD patients and CVD only patients independently. No other significant correlations were observed. *Table 3.4* highlights all correlations performed.

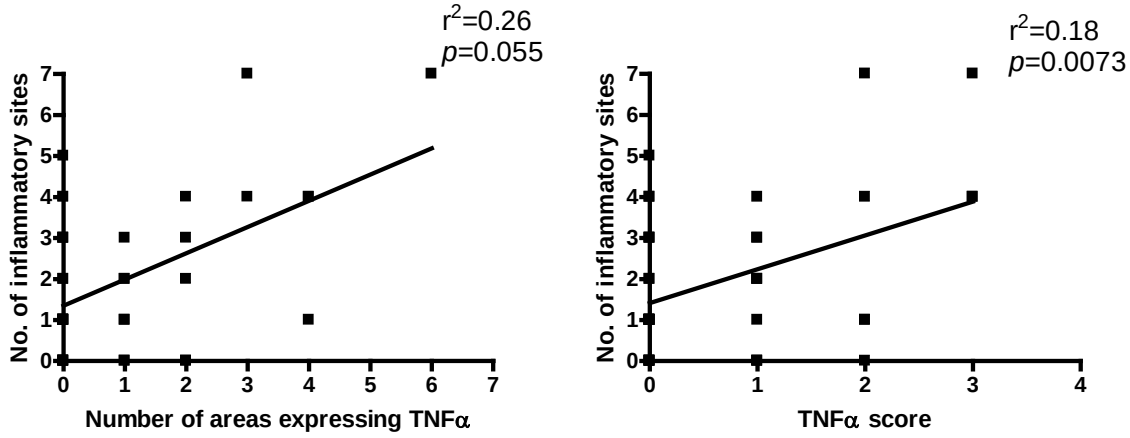


Figure 3.10 TNFα and inflammatory infiltrates in all patients

Levels of adventitial TNFα and number of inflammatory infiltrates were determined in all patients (n=39). The number of inflammatory infiltrates was correlated with the number of independent sites of TNFα expression (p value=0.055 with r^2 0.26) (A) and the TNFα score (p value=0.0073 with $r^2$0.18) (B). P value was calculated by spearman rho and $r^2$by linear regression.

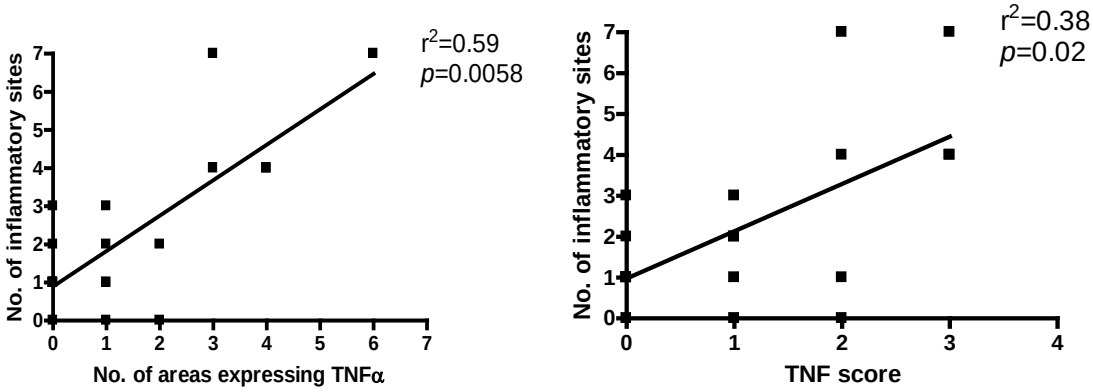


Figure 3.11 TNFα and inflammatory infiltrates in RA+CVD patients

Levels of adventitial TNFα and number of inflammatory infiltrates were determined in RA+CVD patients (n=19). The number of inflammatory infiltrates was correlated with the number of independent sites of TNFα expression (p value=0.0058 with r^2 0.59) (A) and the TNFα score (p value=0.02 with $r^2$0.38) (B). P value was calculated by spearman rho and $r^2$by linear regression.

	All patients (n=39)		CVD only (n=20)		RA + CVD (n=19)	
Predictor	No. of TNFα areas	TNFα score	No. of TNFα areas	TNFα score	No. of TNFα areas	TNFα score
Age	0.34 (ns)	0.53 (ns)	0.25 (ns)	0.22 (ns)	0.58 (ns)	0.89 (ns)
B.M.I	0.39 (ns)	0.54 (ns)	0.41 (ns)	0.47 (ns)	0.97 (ns)	0.88 (ns)
CVD duration	0.17 (ns)	0.17 (ns)	0.76 (ns)	0.82 (ns)	0.18 (ns)	0.26 (ns)
C.R.P	0.06 (ns)	0.02 *	0.37 (ns)	0.43 (ns)	0.29 (ns)	0.12 (ns)
Hypertension	0.34 (ns)	0.36 (ns)	0.18 (ns)	0.19 (ns)	0.48 (ns)	0.45 (ns)
E.S.R	0.37 (ns)	0.31 (ns)	0.46 (ns)	0.39 (ns)	0.89 (ns)	0.96 (ns)

Table 3.4 Predictors of the number of TNFα areas and the extent of TNFα (TNFα score) in the aortic adventitia

*For each section the number of independent TNFα areas was recorded and a score assigned (percentage of smooth muscle cells and inflammatory cells were scored according to the percentage of cells expressing TNFα. 0, negative; 1, >0%<10%; 2, 10-25%; 3, >25%). Spearman's rank correlation coefficient (non-parametric) was used to examine statistical dependence. Non-significant (ns); *p<0.05; **p<0.01; ***p<0.001*

3.2.10 Expression of TNFα in RA+CVD serum

Serum samples were collected from 15 of the 20 CVD patients and all 19 RA+CVD patients (n=19). TNFα levels were measured by Luminex assay and found to be significantly higher in the RA+CVD group compared to the CVD only group [36.6 ± 22 versus 3.7 ± 1.6 pg/ml respectively, *p*=0.0008] (*figure 3.12*). However, converse to expectation, serum TNFα did not positively correlate with TNFα expression in matched aortic adventitia biopsies (*p*=0.10, spearman rho).

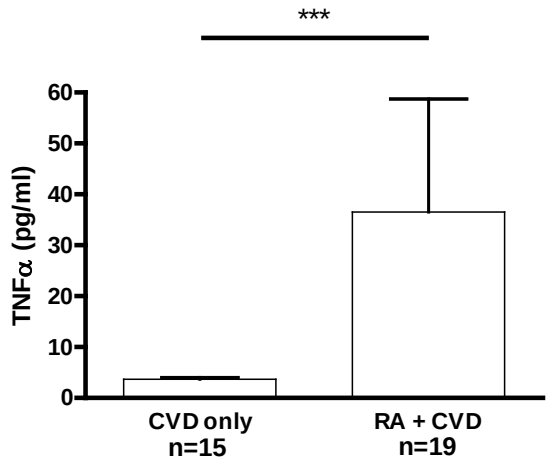


Figure 3.12 Levels of TNFα expression in serum of RA+CVD and CVD only patients

A significant difference was observed between the RA+CVD and CVD only cohort with a mean ± SE of 36.5±22 and 3.7±1.6 pg/ml respectively. p=0.0008 (***) (Mann Whitney U test)

3.2.11 CD21 expression in the aortic adventitia

Having confirmed the presence of inflammatory infiltrate, TNFα and B cells (experiment conducted by Ammad Ahmed 2010) in the aortic adventitia, we next sought to determine whether germinal centres, a site of intense B lymphocyte maturation, were present in the aortic adventitia of patients with CVD. I therefore set out to establish primary follicle/germinal centre staining in human tonsil with an antibody raised against CD21.

To optimise the staining I tried trypsin, citrate and Tris EDTA antigen retrieval with the immPRESS method. Positive staining was not apparent in any of these attempts. I next attempted to use the Avidin/Biotin (ABC) method with a biotinylated secondary antibody. Again attempts with all three antigen retrieval methods similarly resulted in no positive staining. In an effort to further optimise this staining method I altered the primary antibody incubation conditions. Unlike other immunohistochemistry experiments illustrated in this chapter, I did not incubate the samples with the primary antibody at $4^{o}C$ overnight, instead incubating at room temperature for 90 minutes. This approach led to clear cell-specific staining with no background and no signal detectable in the isotype control (*figure 3.13*). The quality of staining was further improved by performing a non-specific block with 10% horse serum, instead of the usual 2.5%, and by incubating the primary antibody in a solution made up of PBS and 2% goat serum.

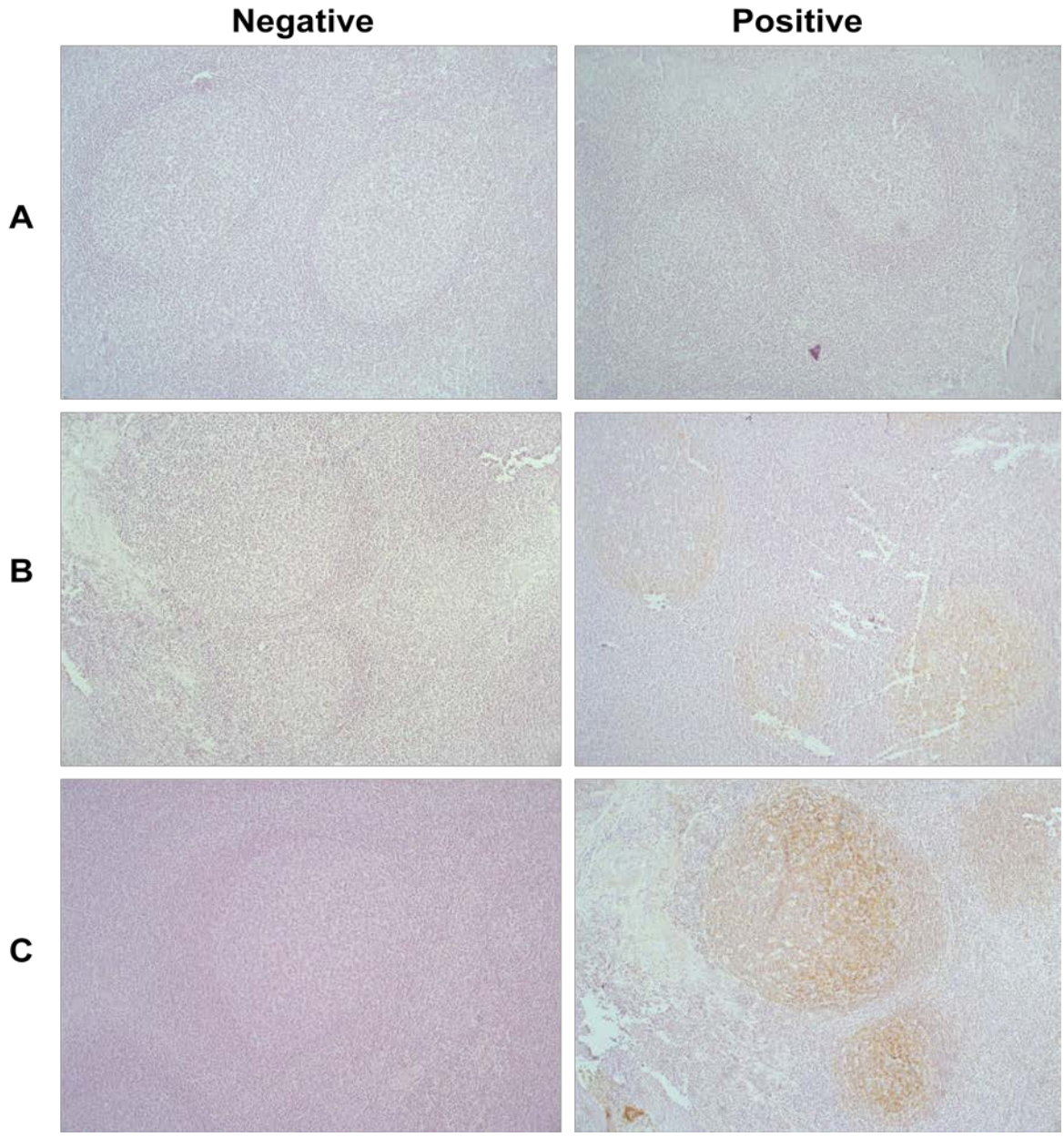


Figure 3.13 CD21 mAb selectively stains germinal centers

In an attempt to detect follicular dendritic meshwork was highlighted by staining for CD21 in human tonsil sections. Isotype controls are shown in the left column (negative). (A) Representative image of failed CD21 staining attempts (100x). (B) CD21 staining using avidin/biotin complex with primary antibody incubation at room temperature for 90 minutes. (C) Same as B with altered serum block and primary antibody buffer conditions.

Germinal centers were detected in tonsil sections and the surrounding follicular mantle zone — the outer ring of lymphatic follicles that surround a germinal centre and contains transient lymphocytes (*figure 3.14*). Dense networks of FDCs were visible in germinal centres and, to a lesser extent, in the follicular mantle.

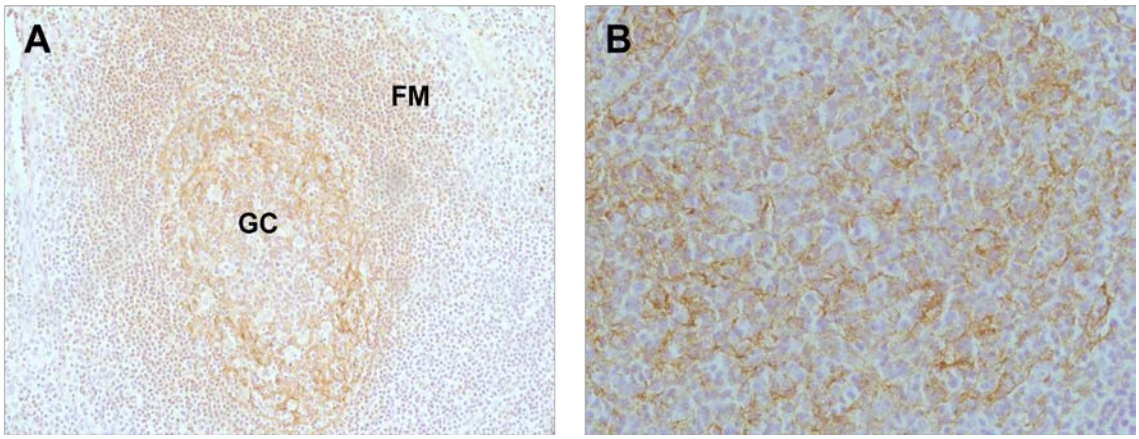


Figure 3.14 mAb CD21 stains tonsillar germinal centres
(A) anti-CD21 staining of FDC networks within a germinal centre (GC) as well as B lymphocytes within the follicular mantle (FM) (100x) (B) High magnification of the germinal centre shown in figure A. FDCs can be distinguished as large cells containing one or two big nuclei and branched projections. Follicular dendritic networks are clearly visible (400x).

We were not able to detect CD21 positive cells in any of the aortic adventitia samples (*figure 3.15*). This interesting observation is surprising as morphology presumed to be follicle-like structures were microscopically observed in several of the aortic adventitia sections (*figure 3.15 D*). However these support the notion that germinal centers are not present in the aortic adventitia.

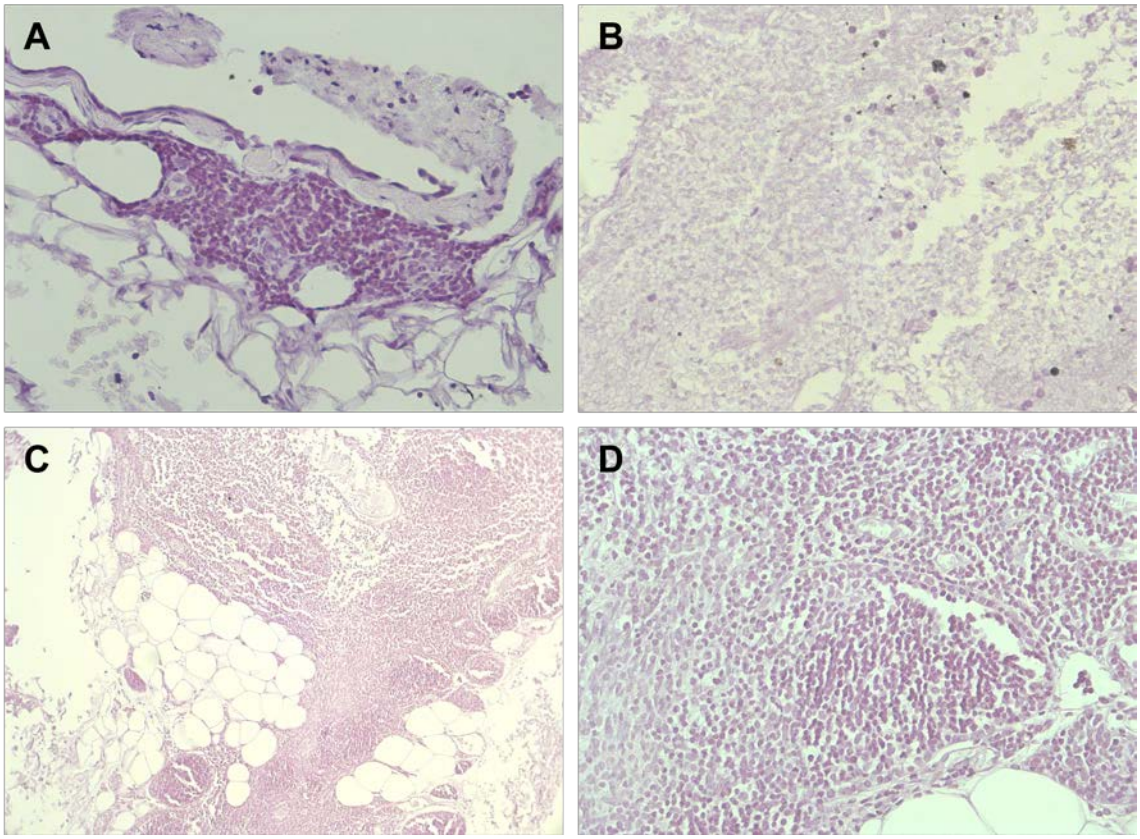


Figure 3.15 CD21staining in aortic adventitia

CD21 was not detectable in the aortic adventitia. Images above are representative of sections that were hypothesised to contain CD21 positive germinal centres. (A) Submesothelial infiltrate (400x) (B) Pronounced adventitial infiltrate (400x) (c) Pronounced adventitial infiltrate with localised areas of dense inflammatory cells (100x) (D) High magnification image of follicle-like structure observed in C (400x).

3.2.12 Immunoreactivity of HSP47 in RA+CVD and CVD only patients

Immunohistochemistry was performed on human aortic adventitia paraffin sections using a monoclonal mouse anti-human antibody raised against HSP47. HSP47 expression was detectable in 13 of the 19 RA+CVD patients (68.4%) and 11 of the 20 CVD only patients (55%). HSP47 was detected in smooth muscle-like cells, endothelial cell, mesothelial cells and the inflammatory infiltrate (*figure 3.16*). No HSP47 staining was observed in adventitial adipocytes.

The extent of HSP47 staining was analysed using a semi-quantitative scoring method in a blinded manner. Each section was imaged by light microscopy and the overall percentage of each cell type to express HSP47 was recorded and assigned a score of 0-3 (no staining, <10%, 10-25%, and >25% respectively).

No significant differences in HSP47 staining between the RA+CVD and CVD only patients was detected in any of the cell populations studied. However, there was a trend towards greater HSP47 expression in smooth muscle cells, mesothelial and endothelial cells in patients who had both RA+CVD compared to CVD only, however these results did not reach significance (*figure 3.17*). We also observed a significant correlation between smooth muscle HSP47 expression and mesothelial HSP47 expression when considering all patients (n=39) (p=0.024, Spearman's rank). The power of this correlation increased when considering CVD only patients (n=20) (p=0.011) but was lost when considering the RA + CVD patients (n=19) (p=0.4).

3.2.13 Relationship between HSP47 expression and traditional CVD risk factors

The relationship between the expression of mesothelial, endothelial, inflammatory cell and smooth muscle cell HSP47 in the aortic adventitia with the following factors was analysed: sex, age, duration of, BMI, CRP, CVD, ESR, and hypertension. When considering all patients (n=39), hypertension positively correlated with smooth muscle cell (p=0.027) and inflammatory cell (p=0.02) HSP47 expression. A positive correlation between mesothelial HSP47 expression and age (p=0.02) was observed when considering all patients and this significance remained when considering the RA+CVD group only (p=0.02). C.R.P positively correlated with mesothelial HSP47 expression in the RA+CVD group only. *Table 3.5* highlights all correlations performed.

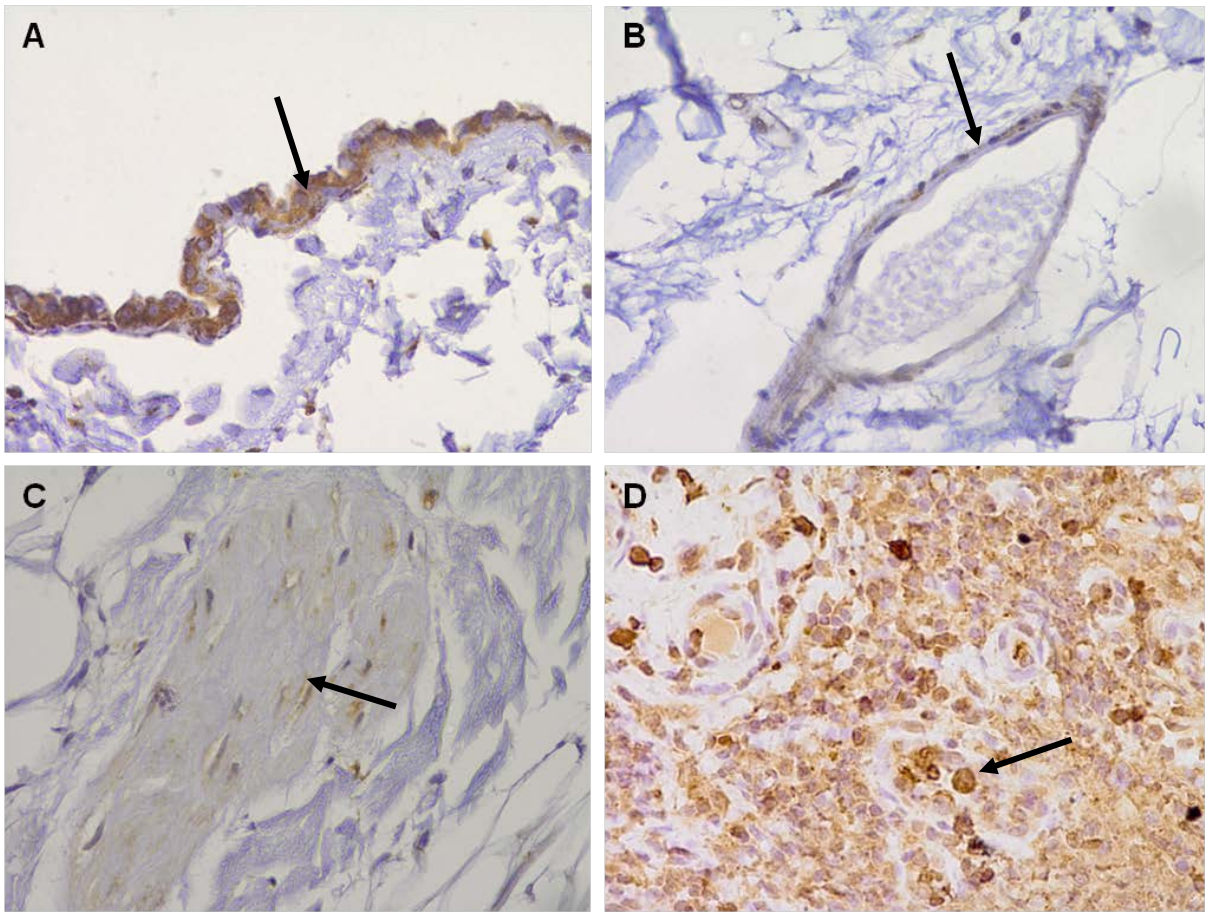


Figure 3.16 HSP47 immunohistochemistry in the aortic adventitia

Representation of typical HSP47 staining in the aortic adventitia. Images were captured by light microscopy at 100x magnification. Cell-specific staining is clearly observable in mesothelial cells (A); endothelial cells (B); smooth muscle cells (C) and mononuclear cell infiltrate (D).

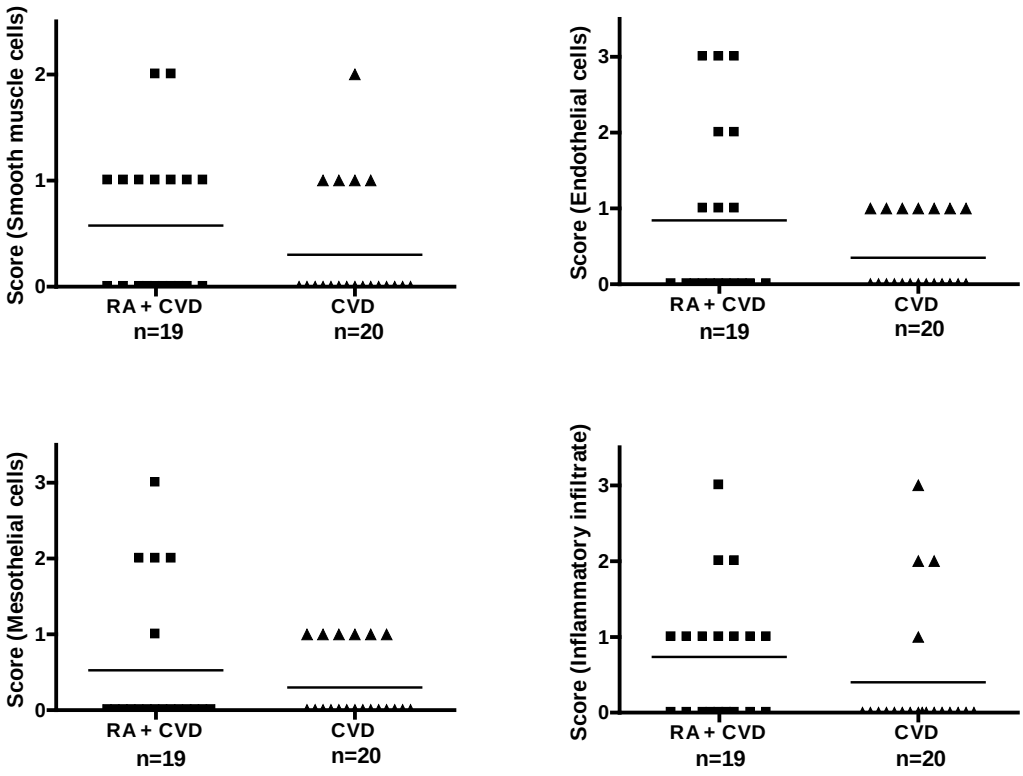


Figure 3.17 HSP47 expression in the aortic adventitia of patients with RA+CVD and CVD only

HSP47 was detected in four independent cell populations (smooth muscle, endothelial, mesothelial and inflammatory cells). Statistical analysis was performed by Mann-Whitney U test for non-parametric data. Y-axis units represent semi-quantitative analysis (0=0%, 1=<10%, 2=10-25%, 3=>25%).

Note: Statistical analysis was also performed by Pearson chi-squared analysis suitable for comparing categorical variables. A significant difference was detected in mesothelial HSP47 expression between RA+CVD and CVD only patients (p=0.022). No significant differences were detected in the remaining cell populations.

	All Patients (n=39)				CVD only (n=20)				RA + CVD (n=19)			
Predictor	Endothelial	Mesothelial	SMC	II	Endothelial	Mesothelial	SMC	II	Endothelial	Mesothelial	SMC	II
Age	0.51	***0.02****	0.21	0.99	0.62	0.49	0.54	0.83	0.64	***0.02****	0.54	0.74
B.M.I	0.25	0.13	0.35	0.55	0.79	0.84	0.58	0.62	0.29	0.13	0.53	0.68
CVD duration	0.70	0.98	0.84	0.28	0.25	0.74	0.56	0.86	0.83	0.83	0.87	0.16
C.R.P	0.34	0.17	0.93	0.41	0.56	0.61	0.79	0.64	0.49	***0.04****	0.92	0.95
Hypertension	0.27	0.41	***0.027****	***0.02****	0.45	0.78	0.41	0.15	0.64	0.41	0.07	0.19
E.S.R	0.25	0.24	0.51	0.382	0.94	0.46	0.93	0.93	0.45	0.15	0.18	0.06

Table 3.5 Predictors of the expression of HSP47 in the aortic adventitia.

*HSP47 expression was recorded as a semi-quantitative score. Spearman's rank correlation coefficient (non-parametric) was used to examine statistical dependence. Non-significant (ns); *p<0.05; **p<0.01; ***p<0.001. Inflammatory infiltrate (II)*

3.2.14 Heat shock protein 60 in the aortic adventitia

Immunohistochemical analysis of human aortic adventitia (n=39) showed no immunoreactive HSP60 in any cases. Clear cell specific staining was observed in the positive control and no background signal was detected in the isotype control (*figure 3.20*).

3.2.15 Calprotectin protein antigen expression in the aortic adventitia

Immunohistochemistry was performed on human aortic adventitia paraffin section (n=39) using a monoclonal mouse anti human antibody raised against calprotectin (antibody kindly donated by Prof. Magne K. Fagerhol, (Ullevaal University Hospital, Oslo). Specifically, aortic adventitial sections were analysed for the s100A9 epitope as this proved to be the most reliable antibody of those available for calprotectin detection.

The calprotectin complex was expressed in areas of mononuclear cell infiltration. Calprotectin expression was detected in inflammatory cells in both the submesothelium and adventitia. Calprotectin was detectable in 8 of the 19 RA+CVD patients (42.1%) and 9 of the 20 CVD only patients (45%). There was no significant difference in either the number of patients expressing calprotectin or the extent of calprotectin expressed across the two cohorts (*figure 3.18*).

Interestingly, very low levels of calprotectin were occasionally detected in smooth muscle or endothelial cells surrounding areas of inflammation. This suggests that neutrophils may be releasing small amounts of calprotectin into surrounding tissue either due to cell disruption or death or as an antimicrobial mechanism. However this hypothesis remains to be investigated.

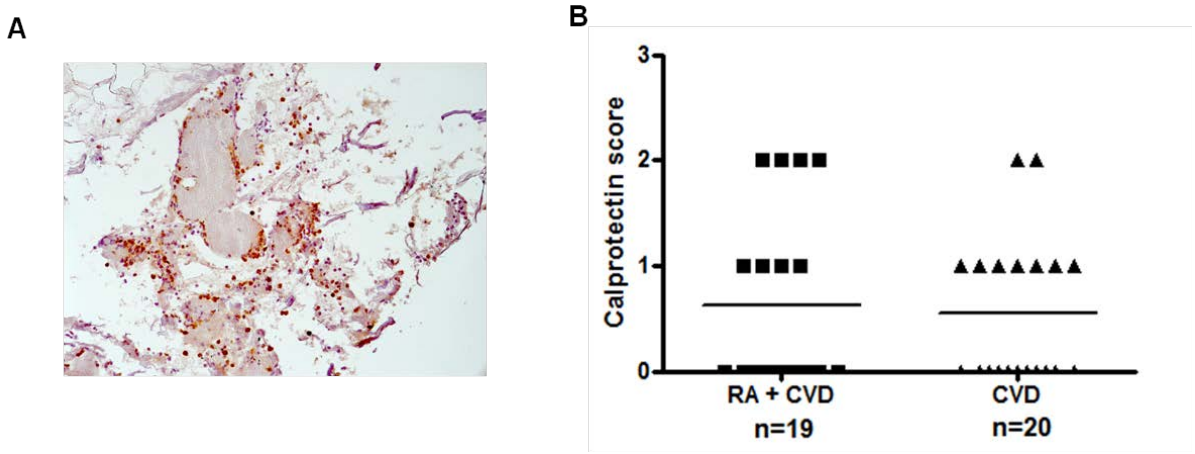


Figure 3.18 Calprotectin in the aortic adventitia

(A) Representative image of calprotectin staining in the inflammatory infiltrate of human aortic adventitia (100x). (B) Semi-quantitative scoring of calprotectin in the aortic adventitia of RA + CVD and CVD only patients. The number of inflammatory cells was approximately counted and each section was scored according to the percentage of cells expressing calprotectin. 0, negative; 1, >0%<10%; 2, 10-25%; 3, >25%. No significant difference was observed between the RA + CVD and CVD only cohort (p>0.05) (Mann Whitney U test).

3.2.16 Calprotectin and TNFα correlation analysis

Previous studies have demonstrated that TNFα can induce calprotectin production from human monocytes (Suryono et al., 2005). In this study, calprotectin expression did not correlate with either the number of independent adventitial TNFα sites or the extent of TNFα expression. Furthermore adventitial calprotectin expression did not correlate with serum TNFα expression (*figure 3.19*).

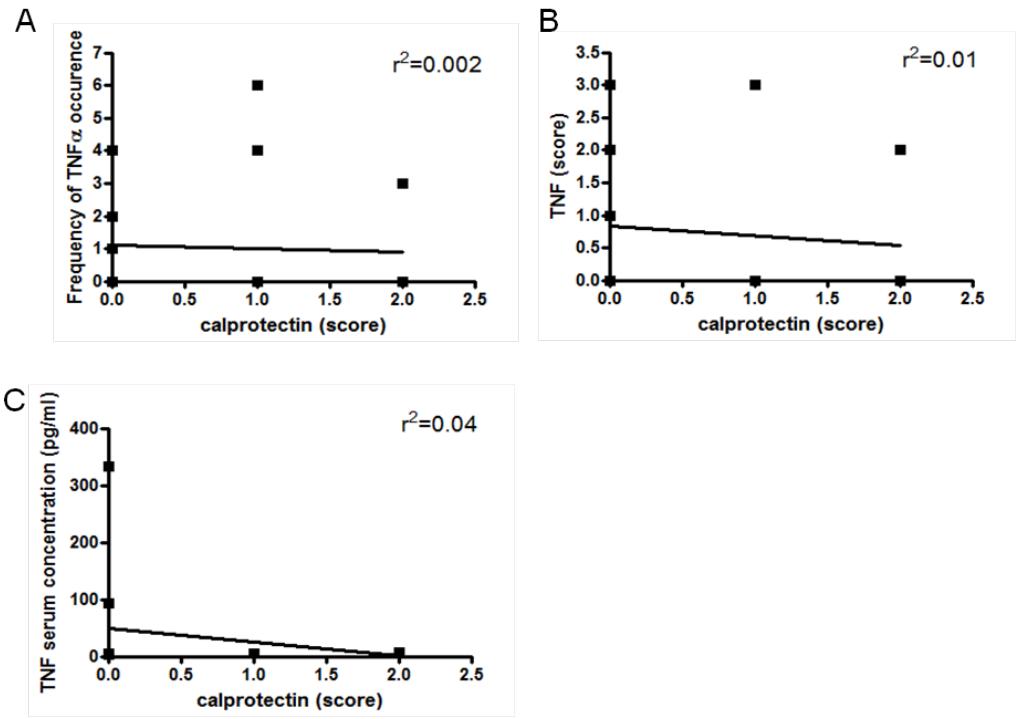


Figure 3.19 Calprotectin and TNFα correlation

Levels of adventitial calprotectin and TNFα were determined by immunohistochemistry in RA+CVD patients and CVD only patients (n=39). Serum TNFα concentration was also determined in 12 of these patients. Correlation analysis was performed for calprotectin expression with: (A) the number of independent adventitial TNFα sites; (B) the extent of TNFα expression (score). Furthermore adventitial calprotectin expression did not correlate with serum TNFα expression. P value was calculated by spearman rho and r^2 by linear regression (all >0.05).

3.2.17 Immunohistochemical quality control

The work outlined in this chapter relied heavily upon immunohistochemistry, and as such the strictest quality control was maintained throughout. All experiments carried out here used monocolonal antibodies (*table 2.1*) which removes the risk of contaminating antibody due to impure antigen used to immunise the host animal.

All experiments, with the exception of the HSP47 immunohistochemistry, carried out on the aortic adventitia sections were first worked up on tonsil sections. HSP47 was worked up on RA synovium sections as these proved to have a source of HSP47 antigen, whereas tonsil tissue did not. Accordingly RA synovium was used as both the positive and negative control. Staining was only performed on aortic adventitia sections once the desired quality of staining was attained and there was no staining in the negative control.

A positive and negative control was carried out in tandem with each experiment. Control images captured from each experiment are provided in *figure 3.20*. In each case, staining is observed in the positive control, whereas no staining is evident in the negative (isotype) control.

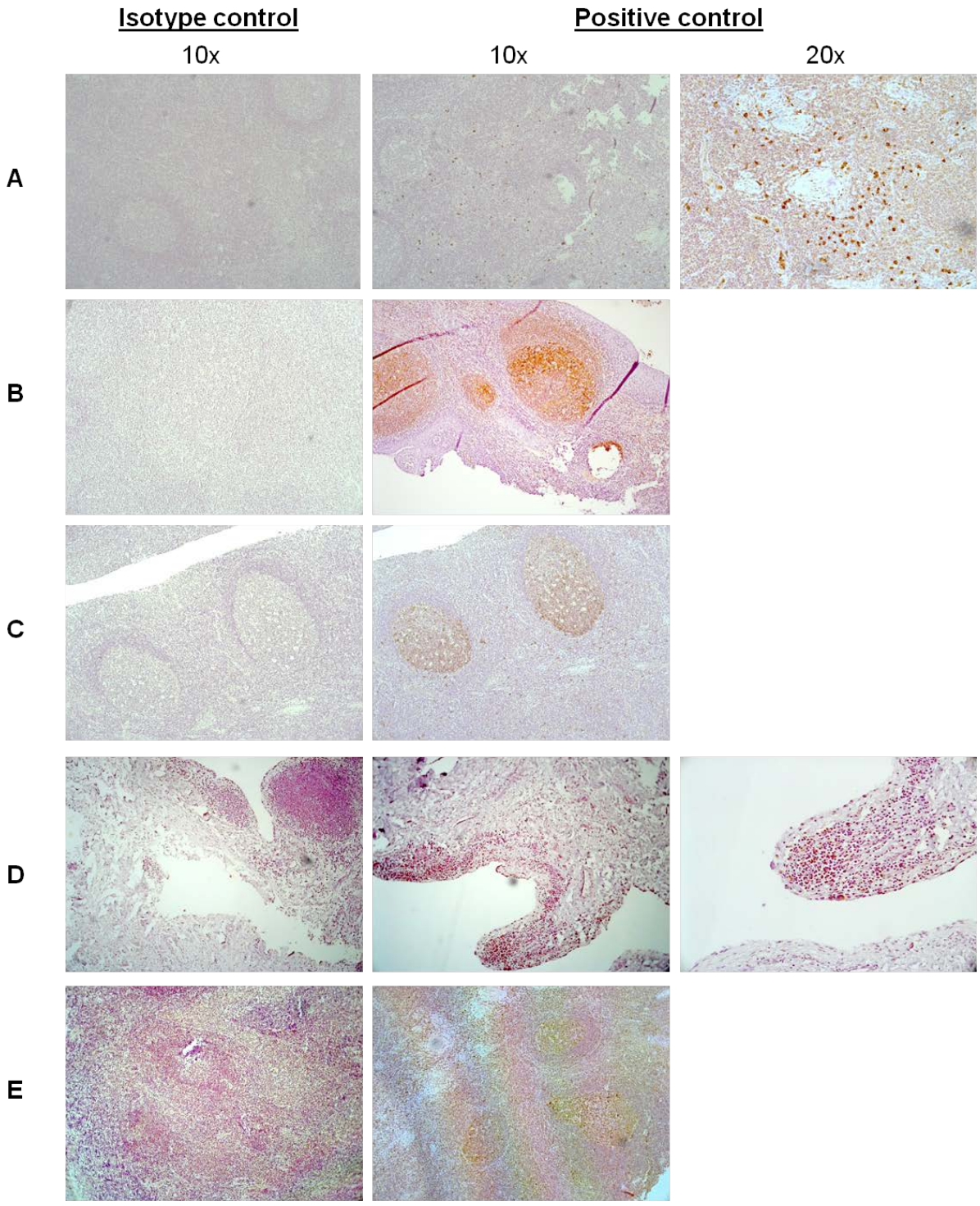


Figure 3.20 Immunohistochemistry quality control

All images were captured from the positive and negative controls carried out in tandem with each experiment (A) calprotectin S100A9; (B) CD21; (C) HSP60; (D) HSP47 and (E) TNFα. Higher magnification of S100A9 and HSP47 has been included for clarity. Control tissue is human tonsil tissue with the exception of HSP47 which is RA synovium..

3.3 Discussion

A growing body of evidence supports the observation that patients with RA experience an increased risk of CVD morbidity and mortality. However, patients with RA do not exhibit a corresponding increase in traditional CVD risk factors that could explain an increased atherosclerotic burden (Wilson, 2008). Evidence exists to suggest that inflammation, being of major importance in the pathogenesis of both RA and CVD, may represent the missing link in explaining the CVD burden observed in RA patients (Aubry et al., 2007, Libby, 2008, Abou-Raya and Abou-Raya, 2006). However, the majority of these studies have concentrated on measuring systemic and general downstream inflammatory markers (e.g. CRP, ESR and total leukocyte counts) as well as traditional inflammatory cytokines (e.g. TNFα, IL-1 and IL-6). To date, only a handful of studies have focused on determining differences in inflammatory profiles between CVD and CVD-RA patients (Hollan et al., 2007, Breland et al., 2010). In the present study we have built upon previous studies by quantifying the extent and incidence of inflammatory infiltration into the aortic adventitia of patients with CVD and CVD+RA.

The aortic adventitia comprises the outermost layer of the aortic wall and has received little attention in research to understand the underlying pathophysiology of atherosclerosis. Additionally, the majority of *ex vivo* human studies on CVD have concentrated on sites of severe atherosclerotic disease. The adventitial sections studied here were taken from areas with reduced macroscopic signs of atherosclerosis. As expected, the atherosclerotic lesion was confined to the intima and on no occasion were the atherosclerotic plaque or lesion observed in the corresponding adventitia.

In this study, we hypothesised that increased aortic inflammation would be observed in patients with both RA and CVD compared to those without RA. When evaluating inflammatory infiltrates in the aortic adventitia we detected inflammation in 18 of the 39 (46%) samples analysed. This data supports descriptions from other authors who have detected inflammatory cells in the outer most layers of the vessel wall. Aubry *et al* (2004) found prominent B-lymphocyte infiltrates in the adventitia of two men with CVD and RA (Aubry et al., 2004). Millonig *et al* (2002) observed inflammatory infiltration in the aortic adventitia of children pre-disposed to atherosclerosis and using immunohistochemistry demonstrated that these were principally composed of T cells and mast cells, (Millonig et al., 2002).

However, in our study, despite the relatively high proportion of patients with adventitial inflammation, no significant difference in either the size or the number of inflammatory sites was observed between the CVD and RA+CVD groups. These findings are in disagreement with Hollan *et al* who examined the severity of inflammatory infiltrates in the aortic adventitia that were removed during routine CABG surgery in 66 patients with inflammatory rheumatic disease and 51 control patients (Hollan et al., 2007). They observed that mononuclear cell infiltrates occurred with greater frequency in the inner adventitia of the rheumatic disease group compared with controls (47% versus 20%; p=0.002). Several factors may explain these conflicting data. Firstly, the study of Hollan *et al* (2007) included a much larger number of patients with a diverse range of rheumatic diseases (including RA, psoriatic arthritis, ankylosing spondylitis, giant cell arteries). Experimental outcome is likely to change when including diseases such as giant cell arteries, as although this is an inflammatory disease of the blood vessels most commonly involving large and medium arteries of the head the disease can involve other large vessels such as the aorta. Furthermore, Hollan *et al* distinguished between inner adventitial infiltrates and infiltrates adjacent to the mesothelium, which they classified as submesothelial (epicardial). As there is no clear anatomic border between epicardial and adventitial loose connective tissue, this differentiation was based on the judgement of pathologists rather than histopathological examination. Due to our relatively small sample numbers and desire to minimise subjectivity we accurately measured total inflammation in each aortic adventitial sample.

The clinical significance of the observed chronic adventitial inflammation remains unknown. Inflammation may have spread from surrounding tissue into the aorta, or may occur *in situ* as a secondary response to atherosclerosis. Alternatively, it may reflect an inflammatory process that is critical to the pathogenesis of atherosclerosis and plaque formation (Higuchi et al., 2002, Scott et al., 1996, Wilcox and Scott, 1996).

In our study, inflammatory infiltration was significantly greater in the aortic adventitia compared to matched internal mammary artery specimens (0.22% ± 0.67and 0.001%±0.003 respectively). As with the aortic adventitia, inflammation in the mammary tissue was observed in and around the perivascular adipose tissue. This is not surprising, as the regulatory effects of adipose cells on inflammation are well characterised, as are the effects of obesity and lipid metabolism on CVD (Ryden et al., 2002, Berg et al., 2004). The very low inflammatory presence in the internal mammary samples supports the fact that this tissue is highly resistant to atherosclerosis. The difference in atherosclerotic

burden between the coronary aortic adventitia and internal mammary artery adventitia may be due to differences in systemic inflammatory mediators, susceptibility to local infection, or due to the presence of autoantigens (HSPs, lipoprotein) at these sites. Unfortunately, owing to sample limitations, we could not investigate these factors. However, future efforts to characterise the atherosclerotic resistance mechanisms of internal mammary artery tissue could have significant therapeutic implications.

Inflammation is a natural defence mechanism against infection and toxic compounds such as lipids and oxidised proteins. Markers of inflammation such as TNFα have been associated with an increased risk for CVD (Ridker, 1997). Furthermore, the pathology of RA is attributable to the pleiotropic biological activities of TNFα (Romas et al., 2002, Keystone, 2001). TNFα is a key cytokine in both the recruitment and activation of inflammatory cells. Furthermore it promotes matrix degradation (Rajavashisth et al., 1999) and thus facilitates the influx of inflammatory cells into the vessel wall.

We set out to ascertain whether TNFα is expressed in the aortic adventitia. This study was principally performed to test the hypothesis that patients with RA would exhibit greater adventitial TNFα expression. TNFα was detected in the aortic adventitia of 12 of the 19 RA+CVD patients (63%) and 6 of the 20 CVD patients (30%), and TNFα expression was significantly greater in the RA+CVD cohort. Elevated TNFα in the aortic adventitia of RA+CVD patients cannot be explained by an excess of inflammatory cells, as we have already demonstrated that there is no difference in inflammatory infiltrate presence between the RA+CVD and CVD groups. However, we have identified that serum TNFα expression is roughly ten times greater in the RA+CVD group compared to the CVD group. Although TNFα is initially synthesised as a transmembrane molecule (Ishisaka et al., 1999), upon cleavage by metalloprotease TNFα converting enzyme (TACE or ADAM17), the secreted monomers that are generated form biologically active homotrimers (Smith and Baglioni, 1987). We hypothesise that elevated levels of secreted TNFα in the serum of the RA+CVD group may infiltrate into and reside in the aortic adventitia, possibly potentiating the onset of CVD in RA. It is worth noting that although there was a trend towards adventitial TNFα correlating with serum TNFα that this did not reach significance. This lack of significance is most likely attributable to our limited sample size.

Sixteen of the 19 RA+CVD patients were taking disease modifying drugs at the time of serum removal and CABG surgery. Only one of these patients was administered anti-TNF

therapy (*etanercept*). However the possibility that other disease modifying drugs may affect TNFα expression cannot be ignored. As most of the other disease modifying drugs (e.g. *methotrexate*, *azathioprine*) administered to RA patients are believed to function by reducing immune cell activation it is highly unlikely that these drugs would cause the elevation of TNFα expression observed in this study. Conversely, it seems highly likely that TNFα expression would be greater in the RA+CVD group if these patients were not confounded by the action of disease modifying drugs.

The overexpression of TNFα in the aortic adventitia of CVD+RA patients suggests that systemically targeted anti-TNFα therapy in RA patients may have beneficial effects in the prevention of CVD and may lead to a reduction in TNFα expression at the sites of atherosclerosis progression. In accordance with these results Jacobson *et al* followed 983 RA patients, 531 who were undergoing anti-TNFα therapy, and measured subsequent CVD events. Their findings suggested that the risk of developing CVD was significantly lower in patients treated with TNF blockers (Jacobsson et al., 2005).

The presence of inflammatory infiltrate, TNFα and B cells (Ahmed., 2010) in the aortic adventitia has been confirmed. In occasional instances, we observed possible nodular lymphoid aggregates which resembled germinal centers in the adventitia. Such structures could accumulate T cells and follicular dendritic cells and support rapid proliferation and differentiation of B cells and corresponding class switch of their antibodies (Perez et al., 2002). To ascertain whether these structures were indeed germinal centres we stained for CD21, as this receptor provides a signal independent of antigen that is required for survival of B cells in the germinal centre (Fischer et al., 1998). In no cases was CD21 detected in the aortic adventitia. This was a surprising result, as CD21 is also involved in the cell activation of B cells and is engaged in homotypic aggregation of B cells (Bjorck et al., 1993). These results are in disagreement with Houtkamp *et al* who identified $CD21^+$ B cells and $CD21^+$ follicular dendritic cells in occasional nodular aggregates resembling lymphoid follicles in the aortic adventitia of CVD patients (Houtkamp et al., 2001). These contrasting results could in part be explained by a recent study by Isnardi *et al* who found that CD21-negative B cells are enriched in patients with autoimmune disease, and that many RA patients also displayed an increased frequency of $CD21^-$ B cells (Isnardi et al.).

Heat shock protein 47 is a stress protein that acts as a molecular chaperone during intracellular processing of procollagen (Nakai et al., 1992). A relationship between HSP47 expression and fibrosis has been identified in human coronary arteries, in which HSP47

was identified in abundance in the fibrous cap of atherosclerotic plaque (Rocnik et al., 2000). Studies also indicate that autoantibodies and inflammatory reactions are directed against surface exposed HSP47 in patients with RA (Hattori et al., 2005). Such studies led us to hypothesise that HSP47 expression may be elevated in the aortic adventitia of patients with both RA and CVD. HSP47 was detected in endothelial, mesothelial, smooth muscle or inflammatory cells. There was no difference between HSP47 expression in endothelial, mesothelial, smooth muscle and inflammatory cells between the CVD and RA+CVD.

As far as we are aware, our study is the first to identify HSP47 in human aortic adventitia tissue. Yokota *et al* have also demonstrated that HSP47 antigen and autoantibody levels are significantly elevated in the serum of RA patients (Yokota et al., 2003). Taken together these findings generate the hypothesis that elevated levels of autoantibodies specific to HSP47 in RA patients may target HSP47 in the aortic adventitia and lead to accelerated atherosclerosis.

When considering all patients (n=39) we observed a strong correlation between smooth muscle and inflammatory cell HSP47 expression and hypertension. Hypertension is associated with structural changes in blood vessels including an increase in the thickness of the media, which is caused by increased cell mass and increased deposition of extracellular matrix. An important component in the latter outcome is collagen, the deposition of which may cause an increase in stiffness of the vessel wall (Deinum, 2002). Thus, it is possible that hypertension may be, in part, due to overexpression of HSP47 and associated collagen accumulation, however this remains to be determined.

Previous studies have demonstrated that human anti-HSP60 autoantibodies are associated with the development of atherosclerosis and can cross react with bacterial HSP60 (Zhu et al., 2001b, Huittinen et al., 2002). Furthermore, subjects with elevated HSP60 in their circulation exhibited vascular dysfunction (Halcox et al., 2005). Experiments in adjuvant-induced arthritis models also suggest that HSP60 may play a vital role in the immune regulation of arthritis (van Eden and Waksman, 2003) and that HSP60 of microbial origins, as well as those of endogenous origins, are targets of immune responses (vanRoon et al., 1997). These studies led us to hypothesise that HSP60 may infiltrate into or reside in the aortic adventitia and could potentially trigger a pro-atherosclerotic response. Converse to this hypothesis, our study of 39 biopsies revealed no HSP60 presence in the aortic

adventitia. These results suggest that HSP60 expression or autoimmunity do not play a pathological role in the adventitia.

Our study was limited to detection of human HSP60, as the only available anti-HSP60 monoclonal antibody did not cross-react with the bacterial homologue. It remains possible, therefore, that bacterial HSP60 may reside in the aortic adventitia and this antigen could potentially trigger a pro-atherogenic immune response.

Several studies on patients with RA have shown strong associations between serum or plasma levels of calprotectin and clinical measurements of joint inflammation (Foell and Roth, 2004, Youssef et al., 1999). Furthermore, Mortensen *et al* observed a negative correlation between plasma calprotectin and HDL, leading the authors to suggest that calprotectin may play a role in atherosclerosis (Mortensen et al., 2009). With this exception, there is no indication that calprotectin has previously been studied in relation to CVD. Our results demonstrate for the first time that calprotectin is expressed in the aortic adventitia and that inflammatory cells are responsible for the production of this peptide.

Calprotectin, a major product of innate immune cells, is an antimicrobial peptide that protects cells and tissues against microorganism invasion and regulates adhesion of leukocytes to the endothelium and extracellular matrix during the inflammatory process (Terrin et al., 2011). The presence of calprotectin in the aortic adventitia, as demonstrated in our study suggests that bacteria may penetrate the vascular wall and reside in the adventitia where they can trigger a calprotectin anti-microbial response. Such an immune reaction could potentiate the onset of atherosclerosis. These findings support one of the most interesting hypotheses of recent years that suggests one or more infectious agents may play a role in atherosclerosis through a direct proinflammatory effect on the vessel wall (Shah, 2001, Leinonen and Saikku, 2002). Further studies to characterise bacterial presence in the aortic adventitia may help explain why calprotectin is expressed in the adventitia and may help to understand the pathophysiology of atherosclerosis.

4 The bacterial composition of the human aortic adventitia: possible implications for inflammatory disease

4.1 Introduction

Premature CVD as a result of advanced atherosclerosis is now understood to be the leading cause of mortality among RA patients (Solomon et al., 2003). Following experiments described in the previous chapter, it was established that the aortic adventitia of CVD patients exhibit extensive inflammatory infiltration. It was also determined that TNFα, a cytokine involved in systemic inflammation that stimulates the acute phase reaction is present in the aortic adventitia. TNFα is released from macrophages in response to stimulation by lipopolysaccahride (LPS) and other bacterial products. Expression of this TNFα was established as being greater in RA + CVD patients than patients with CVD only. It was also observed that calprotectin, a calcium binding protein present predominantly in neutrophils with antimicrobial activity, was detectable in the aortic adventitia of CVD patients in both the presence and absence of RA. Taken together, these findings led to the hypothesis that bacteria observed within the aortic adventitia may generate a pro-atherosclerotic environment.

The mechanisms underlying the pathogenesis of atherosclerosis remain unclear. Recently, infection has received much attention as a potential contributing factor towards atherosclerosis. Specific pathogens have been detected in the arterial vessel wall of CVD patients, and there have been reports of an association between CVD and serological responses to bacterial and viral pathogens such as *Helicobacter pylori, Chlamydia pneumonia*, herpes simplex virus or cytomegalovirus (Saikku et al., 1992, Chiu et al., 1997, Danesh et al., 1999). Furthermore, periodontitis, a disease that results in bacteraemia from oral lesions, has been implicated as a risk factor for the development of atherosclerosis (Haraszthy et al., 2000, Nakib et al., 2004). Lehtiniemi *et al* (2005) observed a diverse bacterial colonisation of atherosclerotic plaques, and consequently hypothesised that atheromas act as mechanical sieves that collect bacteria from the circulation.

To date, most studies have concentrated on *Chlamydia pneumonia* as a possible etiological agent for atherosclerosis (Deniset et al., 2009). *C. pneumonia*-infected endothelial cells are thought to produce reactive oxygen species (ROS), which together with chlamydial antigens cHSP60 and LPS enhance the oxidation of LDL to oxLDL (Kalayoglu et al., 1999). Exposure of macrophages to *C. pneumonia* stimulates the uptake of oxLDL as well as native LDL, which causes macrophages to develop into pro-atherosclerotic lipid-laden foam cells (Kalayoglu and Byrne, 1998). Furthermore, T cells activated by *C. pneumonia*

produce IFN-γ, which activates macrophages and triggers a persistent intracellular infection.

Initial interventional studies with macrolide antibiotics that specifically targeted *Chlamydia* infection after acute coronary syndrome suggested a reduced risk of secondary cardiovascular complications following treatment (Etminan et al., 2004). However, large randomised controlled studies (Azithromycin and Coronary Events Study [ACES], Weekly Intervention With Zithromax Against Atherosclerosis and Related Disorders [WIZARD] and a Randomised Trial of Roxithromycin in Non–Q-Wave Coronary Syndromes [ROXIS]) that tested benefits of treatment with antibiotics such as zithromax and azithromycin indicated no reduction in either CVD or associated complications (Grayston et al., 2005, O'Connor et al., 2003, Gurfinkel et al., 1999).

Despite the major research focus on *C. pneumonia*, several other studies have identified a number of alternative organisms implicated in atherosclerosis. Using 16S ribsosomal RNA (rRNA) molecular sequencing, Marques da Silva *et al.* (2006) detected a wide range of microorganisms in human aortic aneurisms. These included *Stenotrophomonas* spp., *Propioibacterium acnes*, *Brevundimonas diminuta*, *Herbaspirillum* sp., and oral bacteria such as *Streptococcus sanguinis*, *Lactobacillus delbrueckii* and *Tannerella forsythia* (Marques da Silva et al., 2006). However, potential etiological and pathological roles of these bacteria in atherosclerosis remain unclear.

The 16S ribosomal RNA (16S rRNA) is the genetic marker of choice to detect bacteria to the species level. 16S rRNA is a highly conserved region which constitutes part of the 30S subunit of prokaryotic ribosomes. The 16S rRNA gene is a powerful tool for phylogenetic analyses (Janda and Abbott, 2007) as universal PCR primers targeting these conserved regions can be used to amplify the gene in parts to provide the complete nucleotide sequence of the 16S rRNA without prior knowledge of which bacterial species are present. Concomitantly, the 16S rRNA gene also contains hypervariable regions that provide species-specific signature sequences and enable unbiased bacterial identification following sequencing.

Autoimmune rheumatic diseases are generally considered to have a multifactorial aetiology, with susceptibility presumed to be principally due to genetics combined with environmental triggers, of which bacterial infection is considered the most prominent. Most autoimmune diseases, including RA, are associated with specific HLA alleles. Greater than 90% of RA patients possess HLA-DR1 (Ebringer and Wilson, 2000) and

significantly more psoriatic arthritis (PsA) than control patients carry the HLA-Cw*0602 allele (Szczerkowska Dobosz et al., 2005). It has been suggested that the involvement of HLA antigens in the pathogenesis of autoimmune diseases is due to molecular mimicry between certain bacterial antigens and HLA antigens (Wilson et al., 2000).

Psoriatic arthritis is classified as a spondyloarthropathy and is characterised by spondylitis, dactylitis, enthesitis and synovitis, usually manifesting in people with nail and skin psoriasis (Mease, 2010). There is accumulating evidence to support the hypothesis that PsA patients develop CVD prematurely, which may contribute to the early mortality associated with this disease (Wong et al., 1997, Han et al., 2006, Kimhi et al., 2007). Based on current understanding of the pathophysiology of PsA, potential contributors to CVD include inflammation-induced atherosclerosis and metabolic syndrome including hyperlipidiemia, hypertension and obesity (Ku et al., 2009). It has been demonstrated that the use of TNF inhibitors in PsA are associated with a reduction in carotid intima-media thickness and was accompanied by a reduction in inflammatory biomarkers, which is independent of alterations in lipid profile (Mease, 2010).

Although the aetiology of PsA remains unclear, it has been proposed that systemic bacterial infection and/or alteration of the gastrointestinal flora may trigger a Th17 response and increased production of other pro-inflammatory biomarkers that contribute to disease progression. If proved correct, this could help explain why PsA patients have a higher morbidity rate from CVD than the general population.

Considering all the evidence, it is plausible to hypothesise that inflammatory rheumatic disease increases patient susceptibility to infection by specific bacterial pathogens. The objective of the research described in this chapter was firstly to identify the bacteria present in the aortic adventitia of patients with CVD+RA, CVD+PsA and CVD only. The second objective was to characterise the potential atherosclerotic pathogenicity of any species that appeared at an elevated frequency.

4.2 Results

4.2.1 Study plan and patient characteristics

The bacterial diversity of aortic adventia biopsies taken from patients with CVD+RA, CVD+PsA and CVD alone were analysed by amplification of the 16S rRNA gene from total extracted DNA. 16S rRNA gene amplicons were then cloned, sequenced and indentified by GenBank alignment.

All human aortic adventitia (AA) biopsies were from patients who underwent routine coronary artery bypass graft (CABG) surgery, and were provided by the Feiring Heart Biopsy study. All patients were confirmed as free from any clinically significant infection or malignancy prior to CABG surgery. A total of 31 aortic adventitia samples were obtained, from patients diagnosed as having CVD. Of these patients, 11 had coexisting RA, and nine patients had coexisting PsA. The prevalence of known risk factors and the demographic distribution is outlined in *table 4.1*.

Characteristics	**CVD-only (n=11)**	**RA+CVD (n=11)**	**PsA (n=9)**
Age, years	68±8	66±9	61±6
Male sex, no. (%)	4 (36)	4 (36)	5 (55)
Current smokers, no (previous)	0 (6)	2 (5)	4 (2)
C – reactive protein mg/l	3.3±2	10±9	8.3±7
Duration of coronary artery Disease, years	5.1±4	2.6±4	3.7±5
Time from angiography to CABG surgery, days	6.7±8	9.7±8	15±18
Acute coronary syndrome,no (%)	1 (9)	4 (36)	3 (33)
Hypertension, no (%)	8 (72)	4 (36)	5 (55)

Table 4.1 Demographic and clinical characteristics of patients with CVD, RA+CVD and PsA ± SD

4.2.2 DNA extraction and contamination control

To ensure experimental sterility a negative control was processed in parallel with every sample. Furthermore, all reagents used (trizol, chloroform, 100% ethanol, 75% ethanol, 0.1M sodium citrate, isopropanol and DEPC water) were tested for the presence of possible bacterial DNA contamination (*Methods section 2.2.7*). In all cases the negative control did not show any bands. *Figure 4.1* illustrates two 16S PCR samples that were PCR-positive for the presence of the 16S rRNA gene (RA74 in lanes 2-4 and RA63 in lanes 6-8) and the respective positive and negative controls. *Figure 4.2* illustrates that patient RA37 was negative for the presence of the 16S rRNA gene and that bacteria were either absent or below the limit of detection. Furthermore, in consonance with the negative controls, all experimental reagents were demonstrated to be sterile (*figure 4.3*).

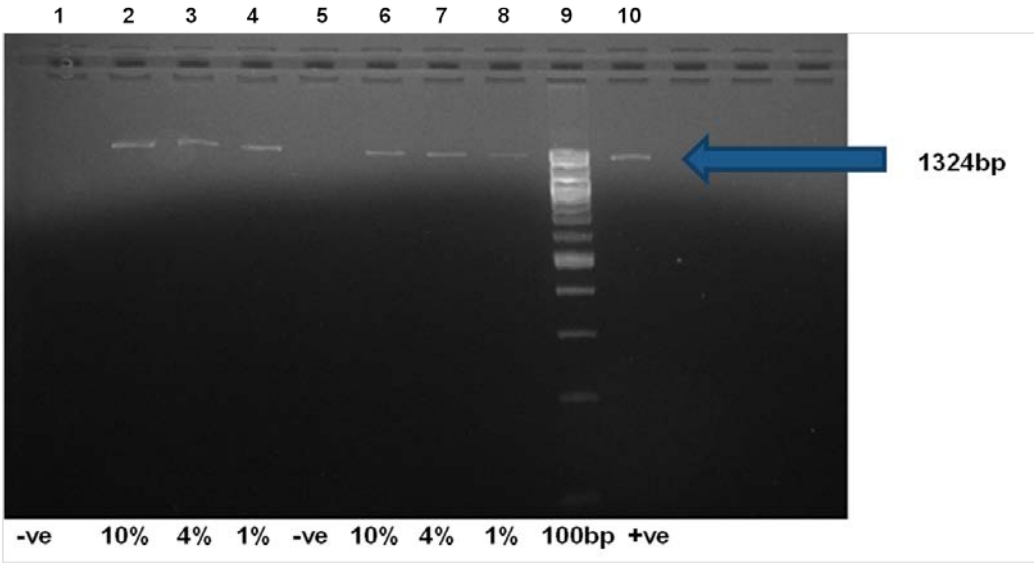


Figure 4.1. Samples PCR-positive for 16S rRNA gene

2% agarose gel electrophoresis of DNA extracted from the aortic adventitia of patients. Each sample was diluted in mastermix (10, 4 and 1%). RA74 (lane 2, 10%; lane 3, 4%; lane 4, 1%) and RA63 (lane 6, 10%; lane 7, 4%; lane 8, 1%). 100-bp DNA ladder (lane 9), negative controls (lanes 1 and 5), positive control (lane 10).

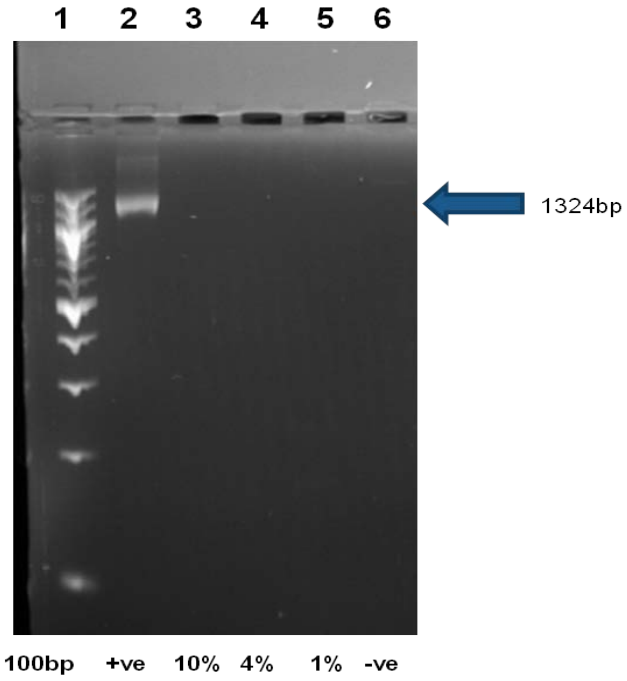


Figure 4.2. Samples PCR-negative for 16S rRNA gene

2% agarose gel electrophoresis of DNA extracted from the aortic adventitia of patients RA37. The sample was diluted in mastermix (10, 4 and 1%). 100-bp DNA ladder (lane 1); positive control (lane 2); negative control (lane 6) and RA37 (lane 3, 10%; lane 4, 4%; lane 5, 1%)

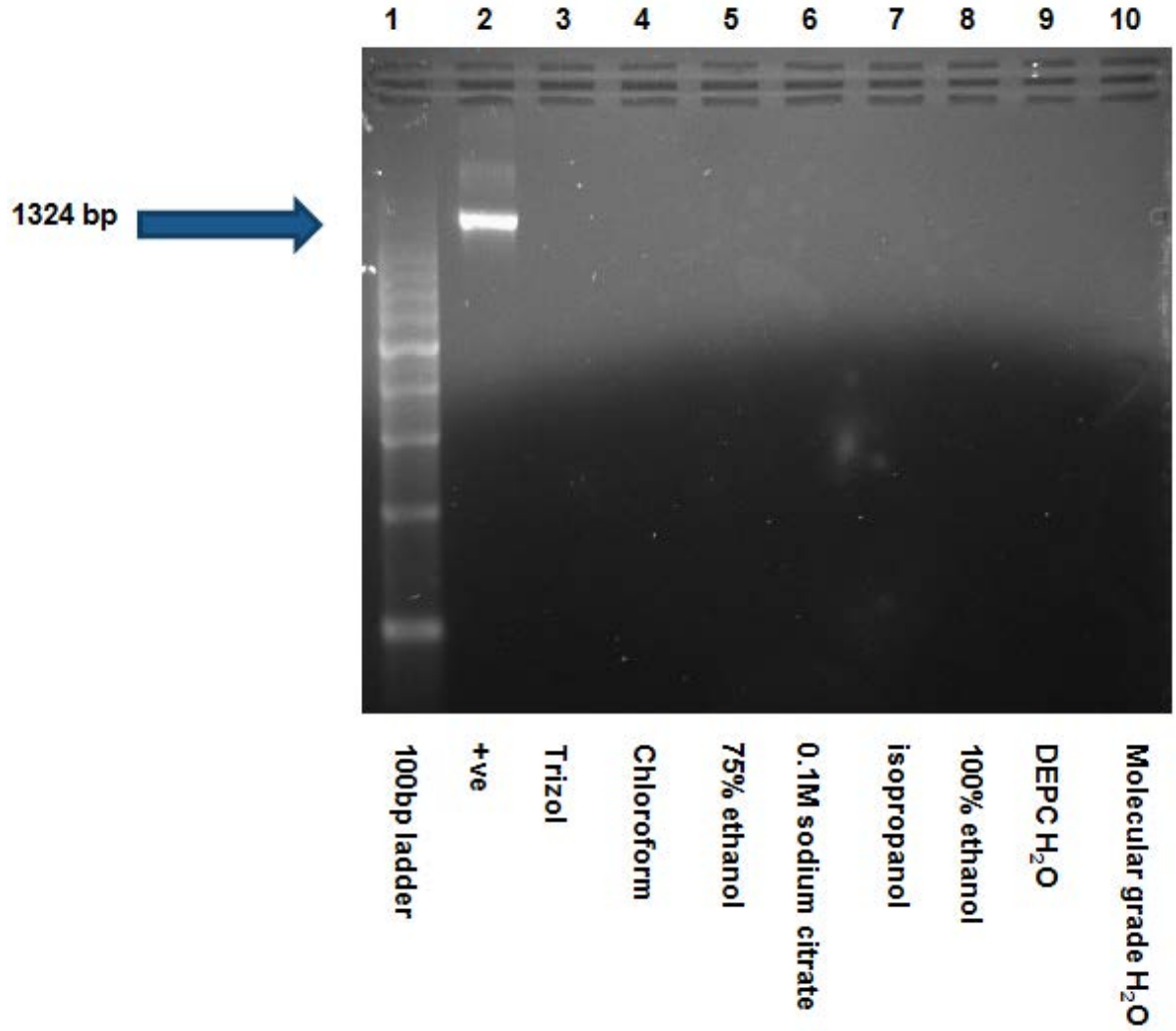


Figure 4.3. Reagent sterility

2% Gel electrophoresis of DNA extracted and 16S rRNA gene PCR amplified from all reagents used in DNA extraction. 100-bp DNA ladder (lane 1); positive control (lane 2); trizol (lane 3); chloroform (lane 4); 75% ethanol (lane 5); 0.1M Sodium Citrate (lane 6); isopropanol (lane 7); 100% ethanol (lane 8); DEPC H_2O (lane 9) & molecular grade water (lane 10)

4.2.3 Bacterial DNA signatures detected in the aortic adventitia of patients with and without RA

Sequencing of a 1324bp region of the 16S rRNA gene was used to identify bacteria present in the aortic adventitia of CVD patients with and without RA. Four of 11 non-RA patients and 3 of 11 RA patients had detectable levels of bacteria present in the aortic adventitia ($p>0.05$), whereas no bacteria were detected in negative controls. All sequences analysed showed >98% similarity to their associated sequences in GenBank. All of the positive specimens contained more than one bacterial species. The four positive samples from non-RA patients harboured a total of 31 different bacterial species (*table 4.2*), and the three positive samples from RA patients contained a total of 11 different bacterial species (*table 4.3*). A Fisher's exact test resulted in a *p-value* <0.00001 indicating that there was a significant difference in the heterogeneity of bacteria present between RA and non-RA patients. A non-RA patient, K53, displayed the most heterogeneity with 10 different bacterial species being detected. Conversely, RA patient RA74 displayed the least heterogeneity, with only two bacterial species detected. *Methylobacterium* sp. was detected in every positive sample from RA patients and represented the most prevalent bacteria in the RA group contributing 35.3% of the total bacteria detected in these samples. *Stenotrophomonas* sp. contributed 29.7% of all bacteria detected in the RA sample group, but was found in only one patient (RA63). Among the non-RA patients *Sphingomonas* sp. was detected in three samples and was the largest contributor to the overall bacterial diversity (16.3%) of the non-RA group (*table 4.3*). Oral *Haemophilus* sp. and *Stenotrophomonas* sp. were the next most commonly detected bacteria in the non-RA group, with *Haemophilus* sp. being detected in two samples and *Stenotrophomonas* sp. making up the majority of bacteria detected in one patient (K40). Other oral bacteria were detected, including *Streptococcus mitis, Porphyromonas endodontalis* and oral *Neisseria* sp. in the non-RA group and *Veillonella* sp. and *Streptococcus sanguinis* in the RA group.

The duration of RA did not significantly alter the risk of bacterial infection in the aortic adventitia (p=0.825). The median duration of RA in this group was 137 months. There were more men ($n=13$) than women ($n=9$) in this study, and chi-squared analysis showed that they fell equally into each of the patient groups. None of the patients that entered this study had aortic aneurysms.

Patient	% of clones	Bacteria	GenBank Accession No.
Non-RA K48	14	Acinetobacter sp.	GU339300.1
	48	Sphingomonas sp.	EF636054.1
	8	Janthinobacterium aqaricidamnosum strain SAFR-022	AY167838.1
	14	Pedobacter sp. TB2-14-II	AY599662.1
	16	Agrobacterium sp.	GU120649.1
K40	50	Stenotrophomonas sp.	GQ416874.1
	2	Rhodococcus sp.	AB547168.1
	6	Methylobacterium oryzae strain 1021b/DCY52	GU294332.1
	30	Pedobacter sp. TB2-14-II	AY599662.1
	2	Bosea thiooxidans KNUC165	DQ424863.1
	8	Pseudomonas fluorescens strain LMG 14571	
	2	Herbaspirillum putei strain AU14404	EU549857.1
K53	10.5	Sphingomonas sp.	EF636054.1
	2.6	Neisseria sp. Oral taxon 014 str. F0314	GQ131417.1
	7.89	Ralstonia pickettii strain MMA-BtI-3 clone FS1	FJ828883.2
	21	Haemophilus sp. Oral clone JM053	AY349380.1
	7.9	Escherichia coli IHE3034	CP001969.1
	18.4	Pectobacterium carotovorum subsp. carotovorum strain Ecc1-16	FJ527466.1
	7.9	Uncultured Phyllobacterium sp. clone PSC19	GU293169.1
	10.5	Methylophilus leisingeri	AB193725.1
	2.6	Bradyrhizobium sp. Pha-1	AF510604.1
	10.5	Uncultured Pseudomonas sp. clone F1Sjun.54	GQ417217.1
K44	42.4	Haemophilus sp. oral clone JM053	AY349380.1
	3.03	Pseudoxanthomonas sp. 11_4K	EF540482.1
	6.06	Kineosporia rhamnosa strain I-132	NR028600.1
	3.03	Uncultured Providencia sp. clone F4feb.62	GQ418015.1
	21.1	Streptococcus mitis bv. 2 strain F0392	GU470907.1
	6.03	Ralstonia pickettii strain MMA-BtI-3	FJ828883.2
	9.09	Porphyromonas endodontalis ATCC 35406	AY253728.1
	6.06	Sphingomonas sp.	EF636054.1
	3.03	Escherichia coli strain AC1	GU594306.1

Table 4.2: Bacterial species detected in the aortic adventitia of CVD only patients

species (at least 98% identity) identified by 16S rRNA gene sequencing of clones from four aortic adventitia biopsies of CVD patients without RA.

Patient	%	Bacteria	Accession No.
RA74	90	Methylobacterium oryzae strain 1021b/DCY52	GU294332.1
	10	Afipia sp.	FJ711197.1
RA63	89.2	Stenotrophomonas sp.	GQ416874.1
	2.7	Streptococcus sanguinis	GU561393.1
	2.7	Methylobacterium oryzae strain 1021b/DCY52	GU591546.1
	5.4	Pedobacter sp. TB2-14-II	AY599662.1
RA73	6.67	Afipia sp.	FJ711197.1
	13.33	Methylobacterium oryzae strain 1021b/DCY52	GU294332.1
	40	Streptococcus sanguinis	GU561393.1
	13.33	Acinetobacter sp.	GU339300.1
	26.6	Veillonella sp. oral clone VeillC3	AY995752.1

Table 4.3: Bacterial species detected in the aortic adventitia of CVD+RA patients

Bacterial species (at least 98% identity) identified by 16S rRNA gene sequencing of clones from three aortic adventitia biopsies of CAD patients with RA

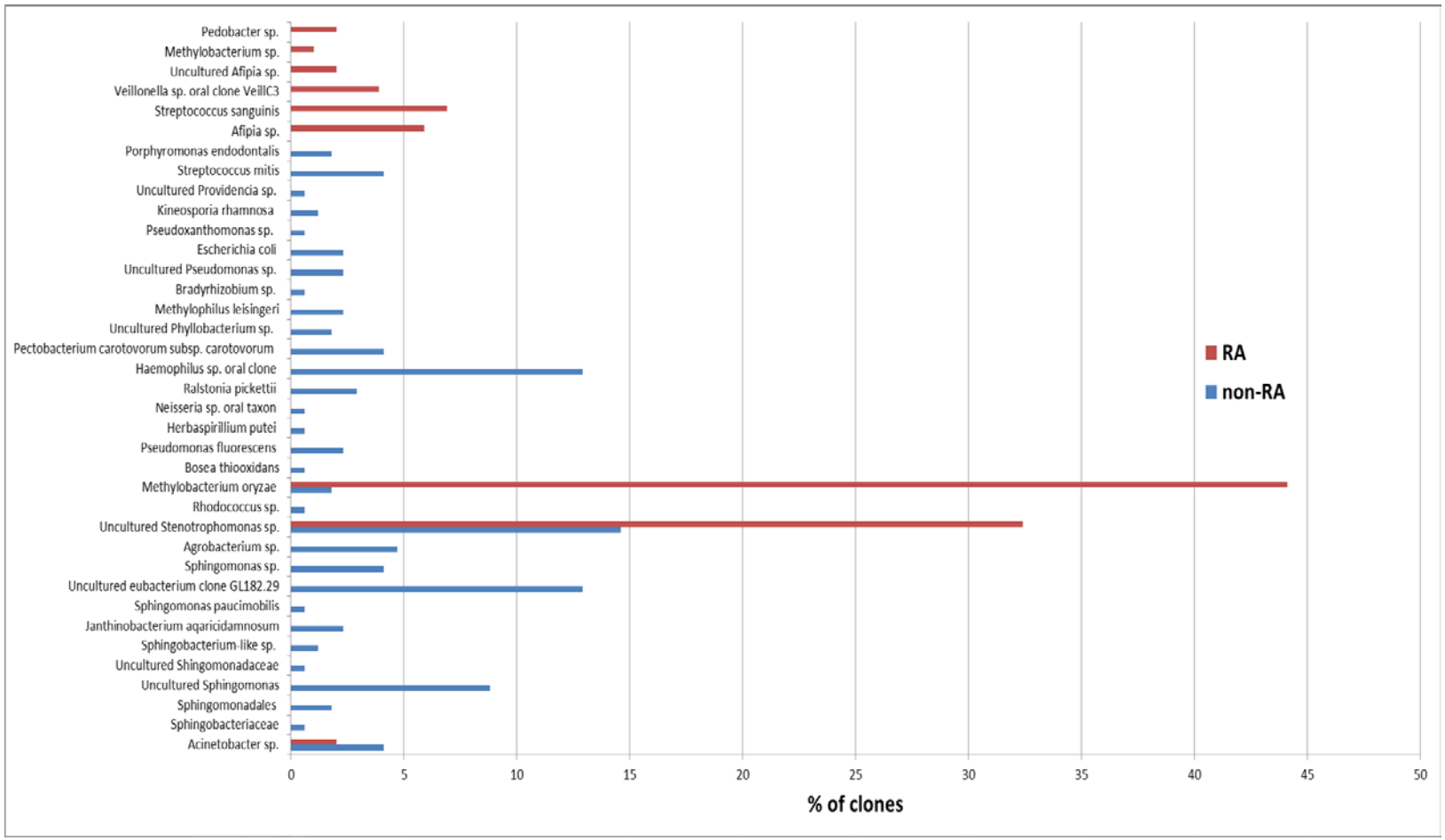


Figure 4.4 Total bacterial species identified by 16S rRNA gene sequencing

Bars represent the percentage of isolated clones which belong to each species in the two cohorts [4 Non-RA (blue bars) and 3 RA patients (red bars)].

4.2.4 Bacterial signatures detected in the aortic adventitia of patients with psoriatic arthritis

The total DNA from the aortic adventitia of 9 patients with PsA was analysed for the presence of the bacterial 16S rRNA gene by PCR. These patients were age- and sex-matched to the 11 control patients (CVD only) described in the previous section. Bacterial DNA was not detectable in any of the adventitial biopsies from the PsA cohort. A Fisher's exact test resulted in a *p-value* <0.00001 indicating there was a significant difference in the heterogeneity of bacteria present in the aortic adventitia between PsA and non-rheumatic patients.

4.2.5 Description of *M. oryzae* bacterium

This study demonstrated that *M. oryzae* was present in the aortic adventitia of all 16S PCR-positive RA+CVD patients. In order to support downstream analysis of host-pathogen interaction it was important to characterise *M. oryzae*. The genus *Methylobacterium* includes a group of Gram-negative, pink-pigmented, facultatively methylotrophic bacteria characterised by their ability to utilise single carbon compounds via the serine pathway, as well as a wide range of multicarbon growth substrates (Madhaiyan et al., 2007). Scanning electron microscopy images demonstrated that *M. oryzae* possess a bacillus-shaped morphology and that the bacterium measures roughly 4.6µm in length and 0.8µm in width (*figure 4.5*). *M. oryzae* occurs singly, in pairs or in rosettes and the cells are convex with regular edges and no flagella. *M. oryzae* grew on both nutrient agar and nutrient agar supplemented with 1% methanol. *M. oryzae* is slow-growing at the optimal temperature of 30^{o}C and takes approximately 35 hours to reach the logarithmic growth phase (*figure 4.6*). Growth was also supported at physiological temperature (37^{o}C) (data not shown).

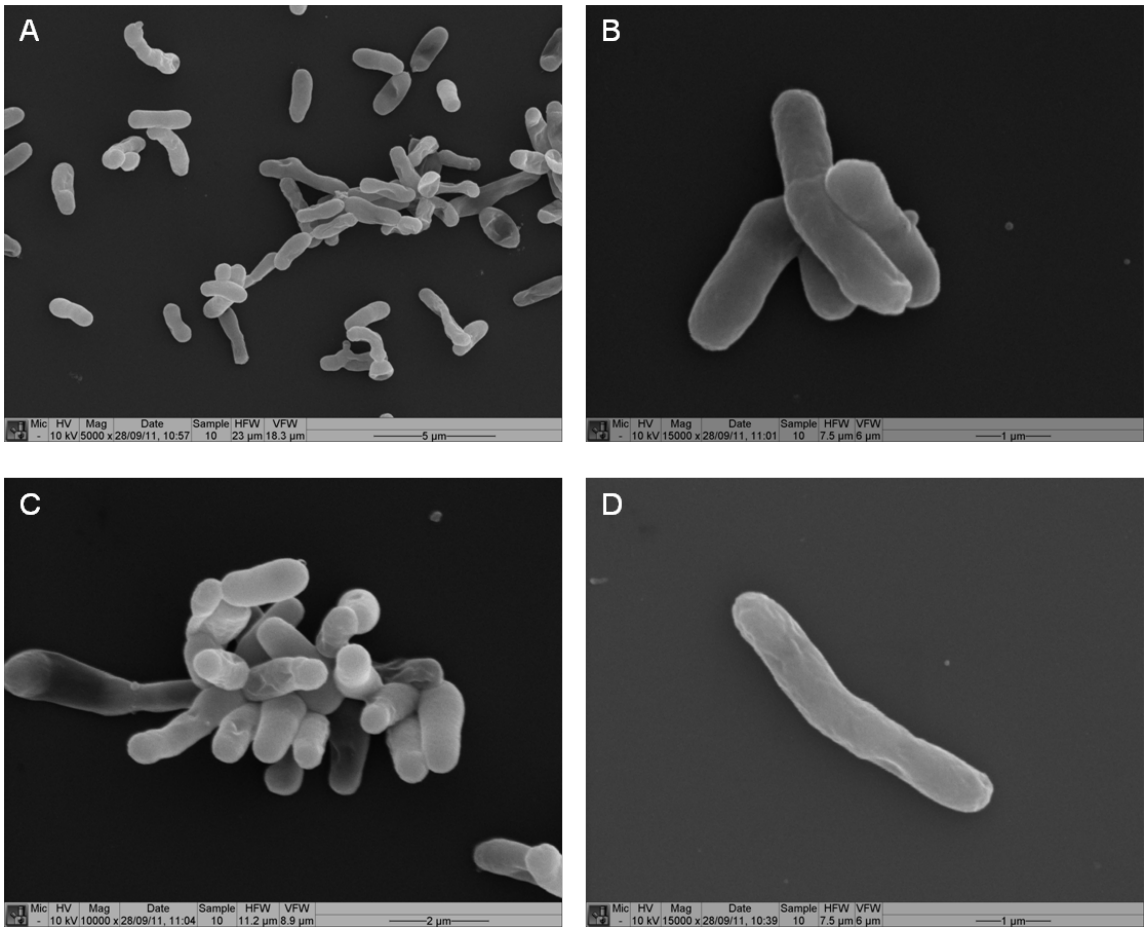


Figure 4.5 Morphological characteristics of M. oryzae

Scanning electron micrographs of M. oryzae: (A) Low magnification view of bacteria (5000x); (B, C) Compact aggregation of M. oryzae (15,000x and 10,000x magnification respectively); (D) Single high magnification micrograph of M. oryzae (15,000x).

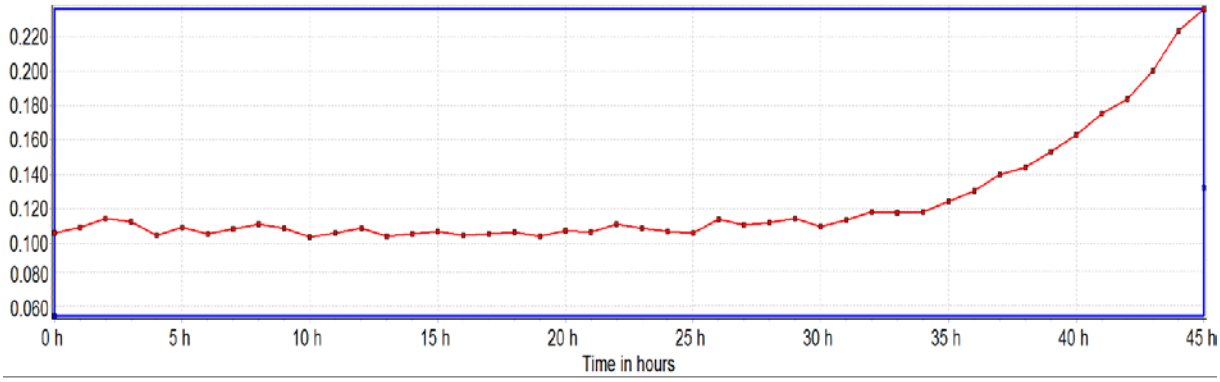


Figure 4.6 Growth rate of M. oryzae

M. oryzae in the logarithmic phase was inoculated into fresh nutrient agar supplemented with methanol and incubated at 30^{o}C. The increase in optical density (OD_{600}) was measured over time.

4.2.6 Broad spectrum analysis of cellular transcription following infection with *Methylobacterium sp.*

The transcriptional profile of atherosclerosis-related genes following infection with *Methylobacterium sp*. was investigated in murine macrophage and endothelial cell lines. This was performed in order to determine if this bacterium could have a pro-atherogenic effect on these cell types.

4.2.6.1 Sequence alignment of 16S rRNA genes of *M. oryzae* and *M. mesophilicum*

At the time of study *M. oryzae* was unavailable, initial experiments in murine cell lines were therefore carried out with *M. mesophilicum*. Prior to experimentation a 16S rRNA gene alignment was performed in order to provide a measure of genetic similarity between *M. mesophilicum* and *M. oryzae* (*See Appendix 5*). A concise linear alignment across the 16S rRNA gene identified a 95.1% genetic similarity, demonstrating that these bacterial species have a highly similar genome and implies very close genetic relationship.

4.2.6.2 Genes analysed by TLDA qPCR array

A qPCR array targeting 90 murine genes involved in immune regulation was used to profile the transcriptional response of murine macrophage and endothelial cell lines to *M. mesophilicum*. Atherosclerosis is a process that involves thickening of vessel walls, a process related to inflammation, which then leads to the formation of plaque. This process is dependent upon proteases, adhesion molecules, growth factors, extracellular matrix proteins and apoptosis signals. A complete list of each of the genes analysed and their properties is provided in the Appendix (*table 6.1-6.8*).

4.2.7 Transcriptional response of murine macrophages to *M. mesophilicum*

Murine RAW 264.7 macrophage cells were infected in duplicate with *M. mesophilicum* at a multiplicity of infection of 200. To profile the cellular atherogenic transcriptional response to infection with *M. mesophilicum* analysis of sequential time points by transcriptional profiling of the 90 genes outlined in *appendix 6* was conducted.

Samples were analysed at four and eight hours post-infection. Each independent sample was normalised to the housekeeping gene *GAPDH* and the transcriptional profile of infected macrophages was determined relative to the vehicle control (*figure 4.7*). A fold change of greater than 1.5, or less than -1.5 was considered biologically relevant. In order to ensure reliability, all replicates with a *Ct* above 33 were excluded in this study.

Measurable levels of transcript was detected from 36 of the 90 genes examined. As expected, gene expression changes were generally amplified with time. Following eight hours of *M. mesophilicum* stimulation, the 10 most strongly upregulated genes and their fold change relative to control were as follows; Ptgs2 (5.1); Csf1 (4.6); Il1β (4.3); Ccl3 (3.8); Fas (3.1); C3 (2.9); Hmox1 (2.7); Cd40 (2.6); Tnfα (2.6) and Cxcl10 (2.0). All of these genes are associated with inflammation: for example, CD40 and Il1β upregulation is characteristic of activated macrophages. The most strongly downregulated gene was IL-15 (-2 fold). Suitably, this gene is expressed by macrophages following viral but not bacterial infection.

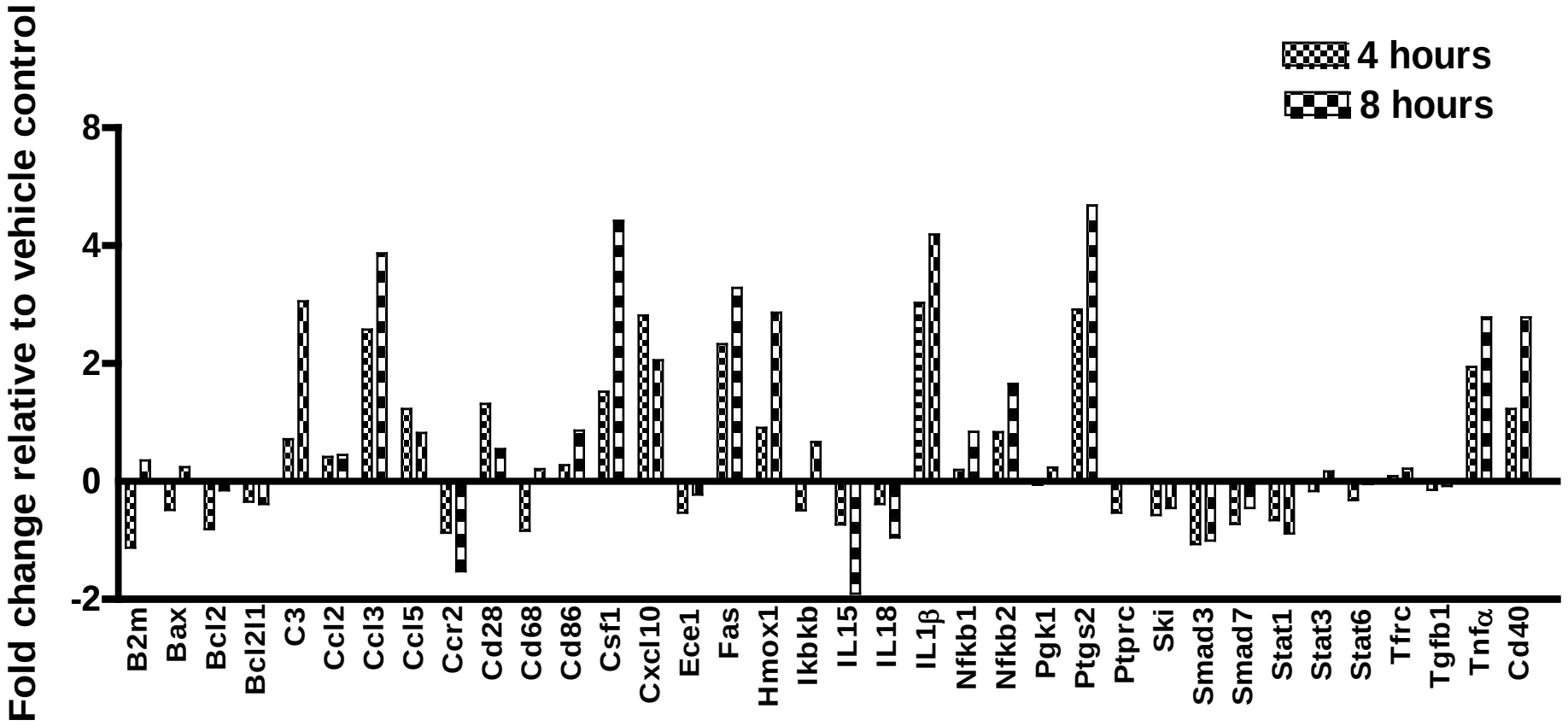


Figure 4.7 Transcriptional profile of RAW 264.7 murine macrophages following M. mesophilicum stimulation *Cells were stimulated with M. mesophilicum at a multiplicity of infection of 200 for four and eight hours. Bars represent experimental duplicates. The fold change for each gene was generated by comparing the means of signal densities of stimulated vs non-stimulated control.*

4.2.8 Biological pathway analysis by Ingenuity IPA analysis of murine macrophages infected with *M. mesophilicum*

Following identification of differentially expressed genes from TLDA experiments, a subsequent post-analysis task was performed in order to find the main biological processes associated with the experimental data. The 'core analysis' function included in IPA (Ingenuity System Inc, USA) was used to interpret the gene expression data for macrophages stimulated for eight hours with *M. mesophilicum* in the context of biological pathways, processes and networks. Both up- and down- regulated genes were defined as value parameters for the analysis and all data with a $Ct \leq 33$ was included. After the analysis, generated biological processes were ordered by a score representing significance.

IPA analysis revealed significant biological functions and canonical pathways to be associated with the differentially expressed genes. The most significant biological function associated with the TLDA-identified genes corresponded to 'inflammatory response' (p=1.32E^{-29} to 1.71E^{-10}) with 29 of the analysed genes being directly implicated in this process. Interestingly, several of the most significant canonical pathways were related to cardiotoxicity [cardiac inflammation (p=5.28E-10 to 1.1E^{-2}), cardiac necrosis/cell death (p=1.24E^{-9} to 3.46E^{-2}) and cardiac dysfunction (7.54E^{-6} to 5.54E^{-3})]. The remaining significant canonical pathways identified were related to renal and liver damage/necrosis.

4.2.9 Transcriptional response of murine endothelial cells to *M. mesophilicum*

Murine SVEC endothelial cells were cultured until 100% confluent and stimulated with exponentially replicating *M. mesophilicum* at an MOI of 200. As in the previous experiment (*Section 4.7*), total cellular RNA was extracted at four and eight hours post-stimulation and the expression profile of 90 genes (*Appendix 6*) analysed by TLDA. Raw fluorescence intensity was processed using the Applied Biosystems RQ data suite. Each independent sample was first normalised to the housekeeping gene GAPDH and the transcriptional profile of infected macrophages was then determined relative to the vehicle control. In order to ensure reliability all replicates with a *Ct* value above 33 were excluded in this study. A resulting fold change of greater than 1.5, or less than -1.5 was considered a biologically relevant change.

Following eight hours stimulation, only five of the 90 genes examined demonstrated a transcriptional fold change of greater than 1.5 or less than -1.5 (*figure 4.8*). *IL6, IL18* and *Ccl2* were upregulated 1.8-, 2.32- and 2.3- fold respectively, whereas *NFkb2* and *Bcl2l1* were downregulated by 1.67- and 1.6- fold, respectively. The majority of genes (79 of 90) were either non-detectable or had a $Ct \geq 33$. Furthermore, mRNA corresponding to *sele* and *selp*, which encode E-selectin and P-selectin respectively, were not detectable following *M. mesophilicum* infection. Upregulation of these genes is a hallmark of endothelial cell activation.

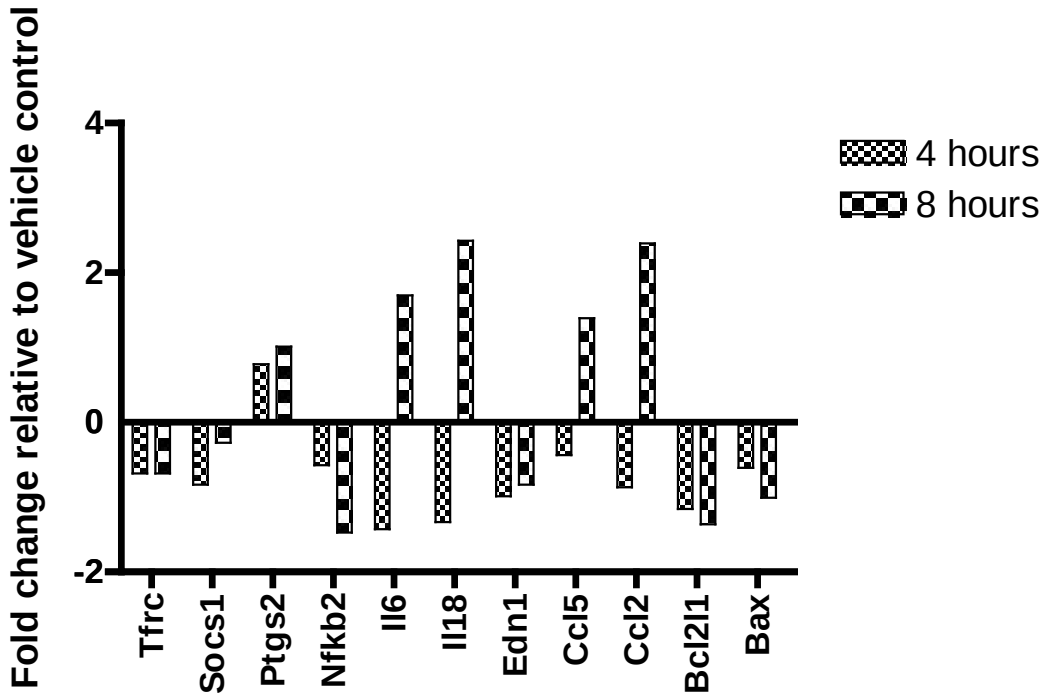


Figure 4.8 Transcriptional profile of SVEC murine endothelial cells following M. mesophilicum stimulation

Cells were stimulated with M. mesophilicum at a multiplicity of infection of 200 for four and eight hours. Bars represent experimental duplicates. The fold change for each gene was generated by comparing the means of signal densities of stimulated vs non-stimulated control.

4.2.10 Immune responses of human primary macrophages to *Methylobacterium oryzae in vitro*

In *Section 4.3* it was shown that *M. oryzae* was detected in the aortic adventitia of 100% of 16S rRNA gene PCR-positive RA+CVD patients and contributed 35% to total bacterial burden. Initial experiments in murine macrophage cell lines demonstrated that *M. mesophilicum* (closely related to *M. oryzae*) triggered a pro-inflammatory response (*see Section 4.2.7*). In order to increase our understanding of how bacteria may behave during atherosclerosis and to increase clinical applicability we next challenged human primary macrophages with *M. oryzae*.

4.2.10.1 Isolation and purity of human primary macrophages

Peripheral blood mononuclear cells (PBMCs) were obtained from the blood of 3 healthy donors. Primary cultures of $CD14^+$ monocytes were prepared using anti-CD14-conjugated magnetic beads. Prior to culture, flow cytometry analysis was performed to assess the purity of $CD14^+$ monocytes. In all cases purity was in the region of 95% (*figure 4.9*). Highly purified adherent $CD14^+$ monocytes were then cultured for seven days in the presence of 15ng/ml M-CSF to ensure monocyte differentiation into macrophages.

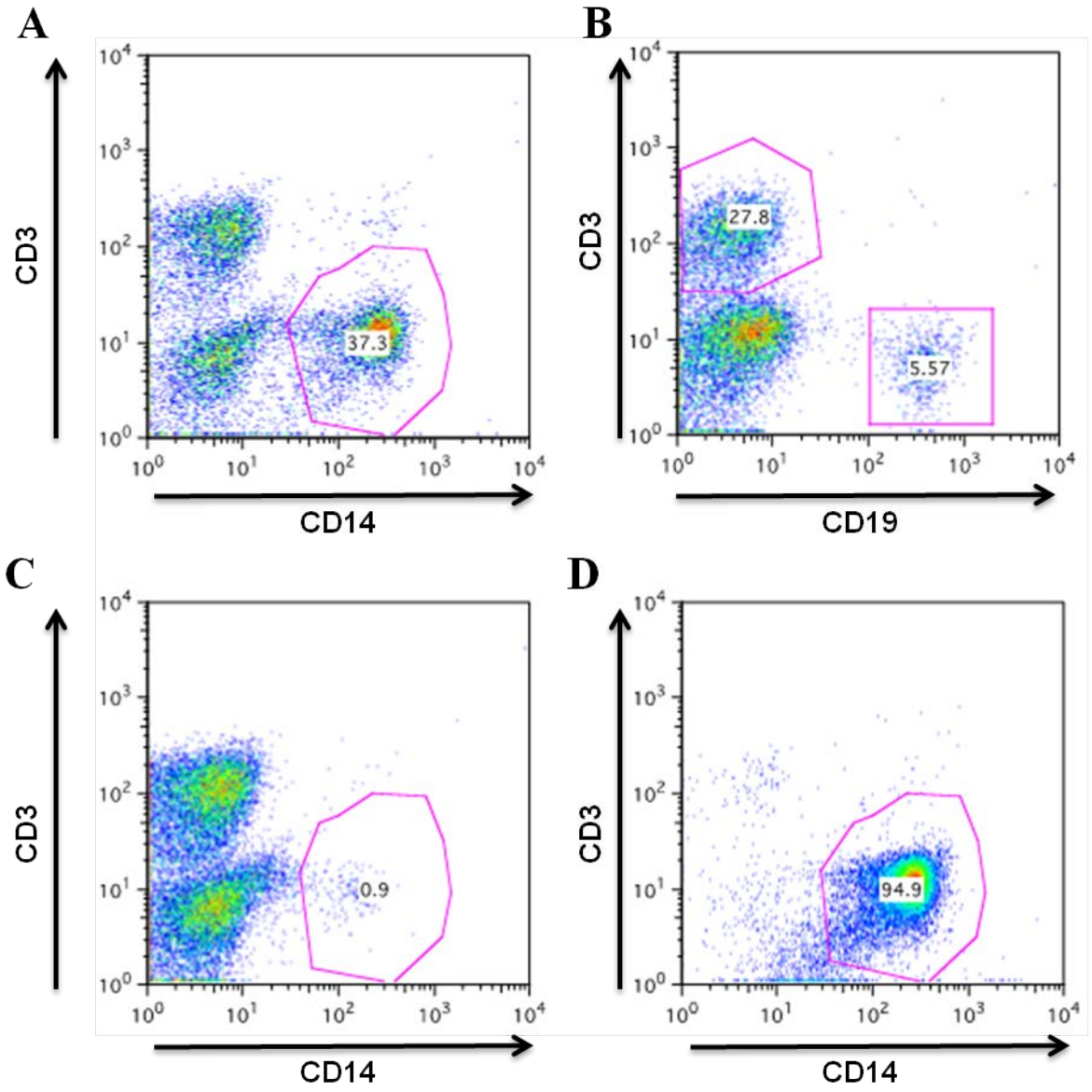


Figure 4.9 FACS plots illustrating purity of isolated CD14+ monocytes

(A, B) FACS analysis on PBMCs prior to $CD14^+$ isolation demonstrates that 37.3% of cells were $CD14^+$ monocytes, 27.8% CD3+ T cells and 5.57% CD19+ B cells. (C) FACS analysis of PBMCs following CD14 separation showing that only 0.9% $CD14^+$ cells remained. (D) FACS analysis following CD14 separation demonstrating that isolated monocytes were 94.9% pure.

4.2.10.2 Transcriptional analysis of human primary macrophages infected with *Methylobacterium oryzae*

Analysis of the relative expression of 90 genes involved in immune regulation (*Appendix: table 6.1-6.8*) showed that, following *in vitro* infection with *M. oryzae*, 40 genes were differentially expressed in human primary macrophages. Thirty-eight of the differentially expressed genes were upregulated and two genes were down-regulated. In general, following 4 and 8 hours of infection with *M. oryzae*, macrophages displayed an excessive pro-inflammatory response.

To facilitate analysis, the differentially expressed genes were divided into several categories based on biological activity. For completeness, *figures 4.10* to *4.16* list all the genes that were detected in primary macrophages following *M. oryzae* infection (*Ct* ≤33). However, the 40 genes which demonstrated at least two-fold increase or decrease in expression in infected macrophages relative to uninfected control cells are highlighted (#).

After both four and eight hours of infection, many of the upregulated genes were those that encode pro-inflammatory cytokines (IL-1α, IL-1β, IL-6 and TNF-α) (*figure 4.10*) and chemokines (CCL3, CCL5, CCL19 and IL-8) (*figure 4.12*). Interestingly, the gene encoding the anti-inflammatory cytokine IL-10 was also induced upon stimulation with *M. oryzae* (62-fold increase after eight hour stimulation). Of the cytokines, IL-18 remained unchanged. IL-18 is a pro-inflammatory cytokine principally produced by macrophages following infection with microbial products such as lipopolysaccahride (LPS).

A set of genes up-regulated in response to the infection encoded co-stimulatory molecules involved in the induction of antigen-specific immune responses (CD40 and CD80) and cell adhesion molecules that are critical for immune cells to enter sites of infection (ICAM-1). Elevated mRNA levels were also observed for apoptosis-regulating genes (Bcl2 and Bcl2l1, showing 12- and 7-fold up-regulation, respectively, following eight hour bacterial stimulation); however mRNA levels of the pro-apoptotic gene Bax remained unchanged.

Granzyme B (encoded by *GZMB*) is a serine protease that is highly expressed in cytotoxic T cells and natural killer cells. The principal function of granzymes is to induce the death of virus infected and other transformed cells. Following infection for four and eight hours,

GZMB was greatly increased in infected macrophages (24-fold at four hours and 197- fold at eight hours). Granzyme B is discussed further in the general discussion (*chapter 6*). Among other genes whose expression was greatly increased was the pro-vasoconstriction agent endothelin 1(*EDN1*) and the enzyme *PTGS2*. PTGS2 converts arachidonic acid to prostaglandin endoperoxide H2 and is the target of the NSAIDs used for the treatment of RA.

Of the 90 genes analysed, *CSF2 (GM-CSF)* exhibited the greatest upregulation (23,000 and 11,000 fold upregulation following four and eight hours stimulation respectively). CSF2 is a cytokine that functions as a white blood cell growth factor which leads to rapid increase in macrophage number *in vivo*. Conversely, the gene encoding the transcription factor SMAD3 (modulates signals of activin and is essential for TGF-beta-mediated immune suppression) exhibited the greatest down regulation following infection (9- and 12-fold down regulation following four and eight hours stimulation respectively).

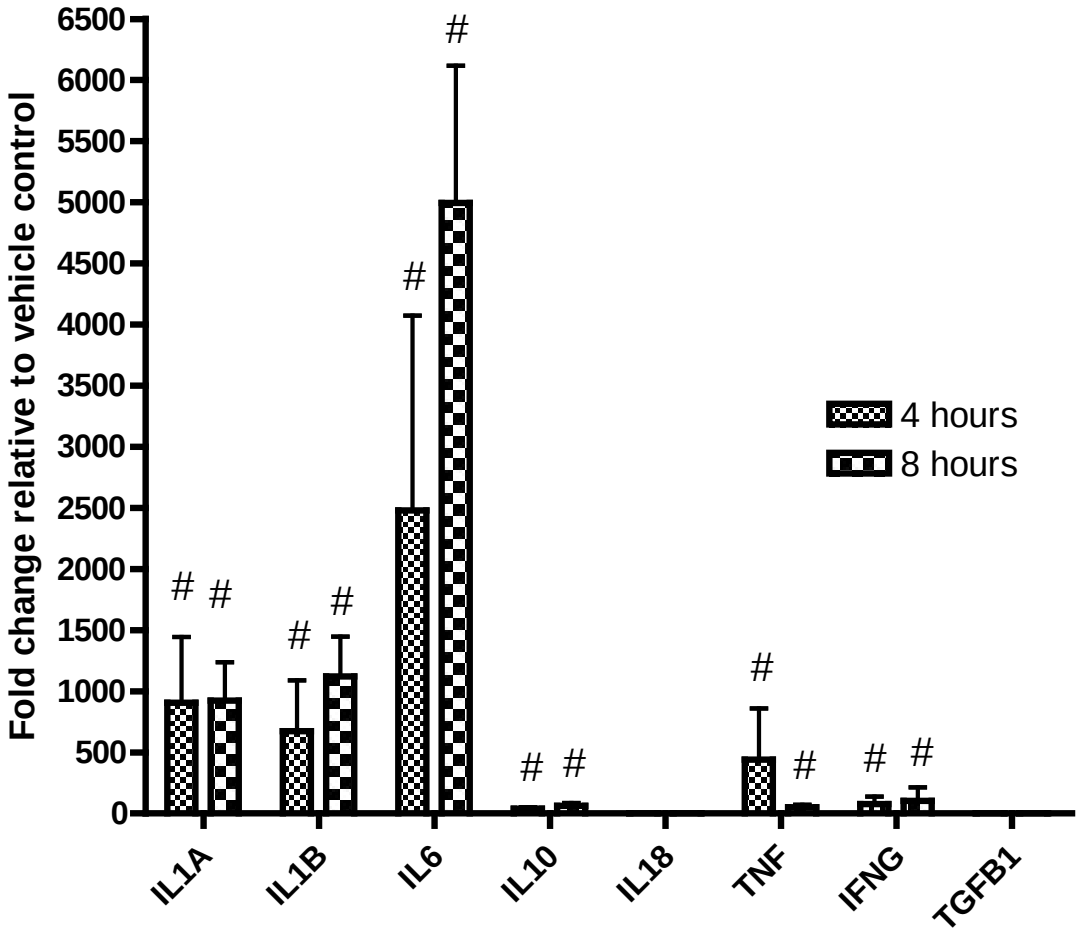


Figure 4.10 Transcriptional profile of genes encoding cytokines in primary human macrophages following M. oryzae stimulation

Primary human macrophages were stimulated with M. oryzae at a multiplicity of infection of 200 for four and eight hours. Each column represents the mean ± standard error of three experiments. The fold change of each transcript was generated by comparing the means of signal densities of stimulated vs non-stimulated control. Fold change >2 (#).

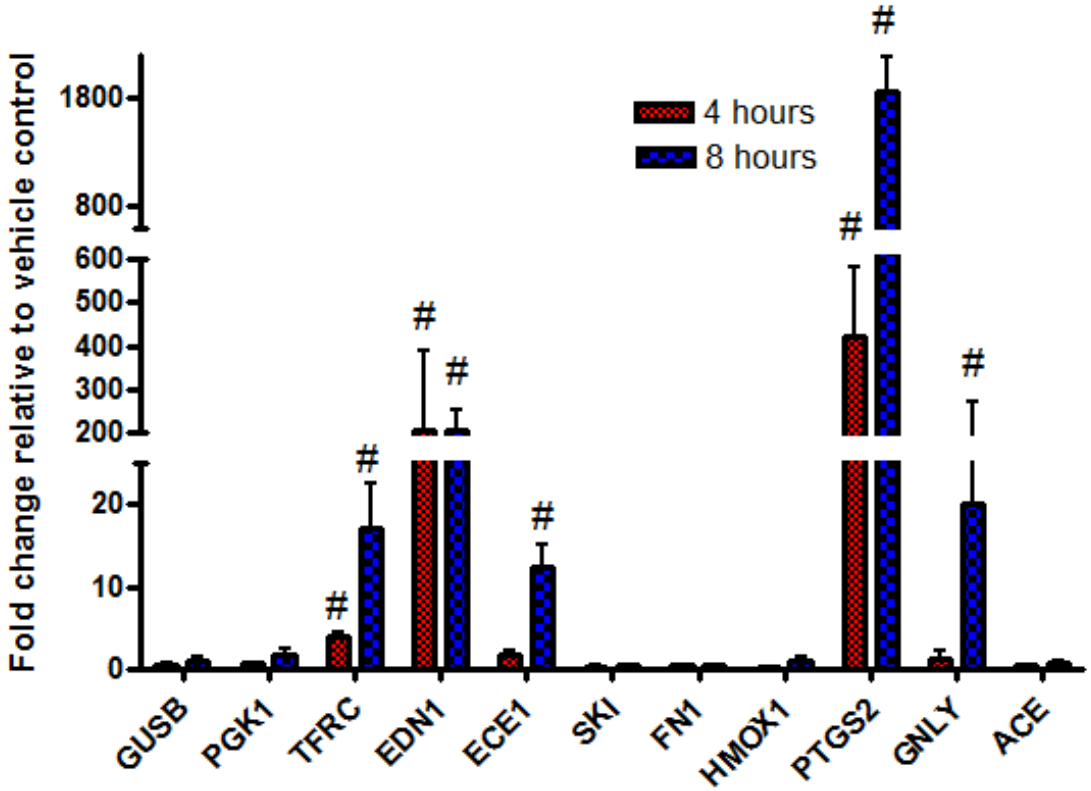


Figure 4.11 Transcriptional profile of genes encoding enzymes or intracellular proteins in primary human macrophages following M. oryzae stimulation

Primary human macrophages were stimulated with M. oryzae at a multiplicity of infection of 200 for four and eight hours. Each column represents the mean ± standard error of three experiments. The fold change of each transcript was generated by comparing the means of signal densities of stimulated vs non-stimulated control. Fold change >2 (#).

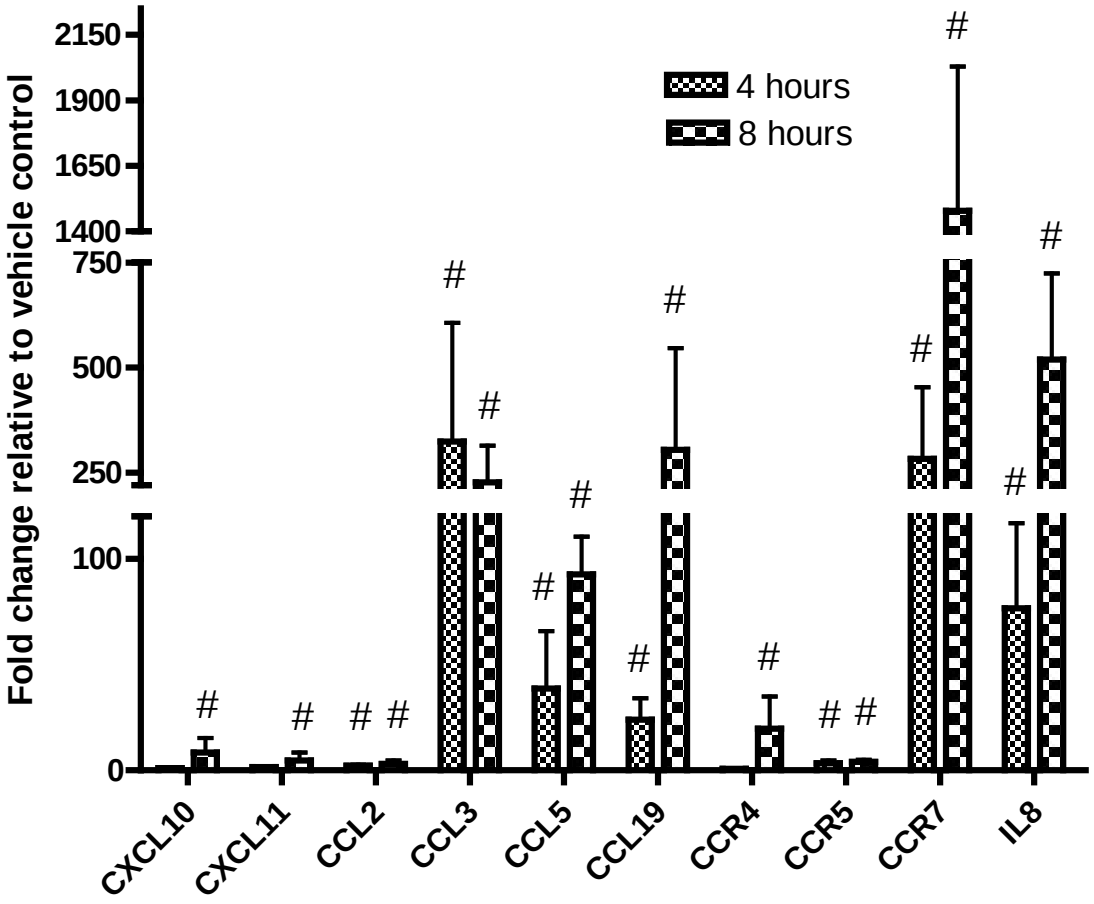


Figure 4.12 Transcriptional profile of genes encoding chemokines in primary human macrophages following M. oryzae stimulation

Primary human macrophages were stimulated with M. oryzae at a multiplicity of infection of 200 for four and eight hours. Each column represents the mean ± standard error of three experiments. The fold change of each transcript was generated by comparing the means of signal densities of stimulated vs non-stimulated control. Fold change >2 (#).

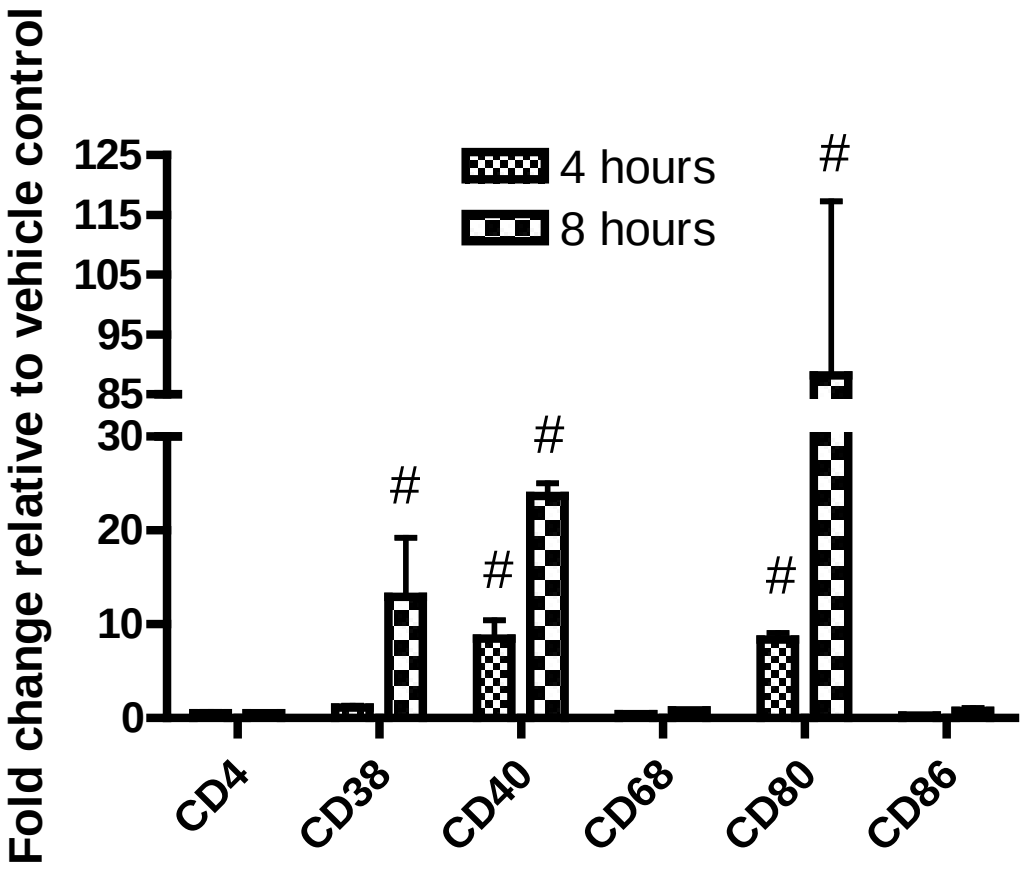


Figure 4.13 Transcriptional profile of genes encoding lineage markers in primary human macrophages following M. oryzae stimulation

Primary human macrophages were stimulated with M. oryzae at a multiplicity of infection of 200 for four and eight hours. Each column represents the mean ± standard error of three experiments. The fold change of each transcript was generated by comparing the means of signal densities of stimulated vs non-stimulated control. Fold change >2 (#).

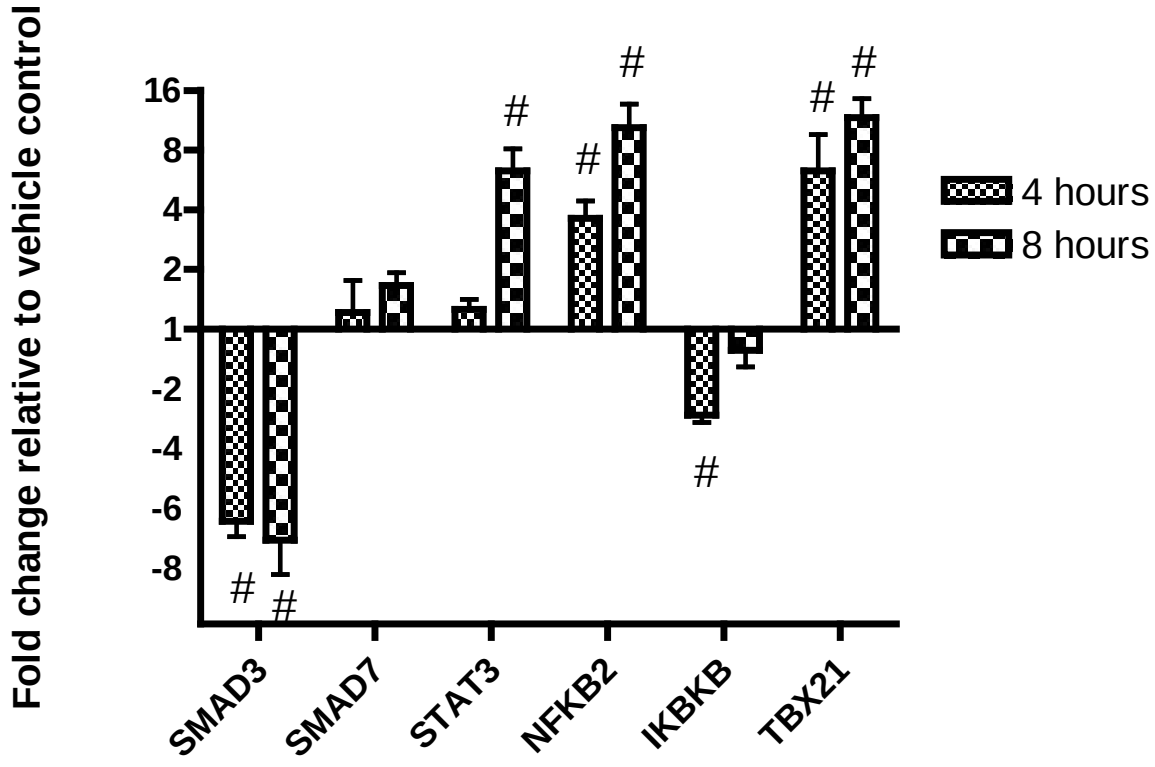


Figure 4.14 Transcriptional profile of genes encoding signalling and transcription factors in primary human macrophages following M. oryzae stimulation

Primary human macrophages were stimulated with M. oryzae at a multiplicity of infection of 200 for four and eight hours. Each column represents the mean ± standard error of three experiments. The fold change of each transcript was generated by comparing the means of signal densities of stimulated vs non-stimulated control. Fold change >2 (#).

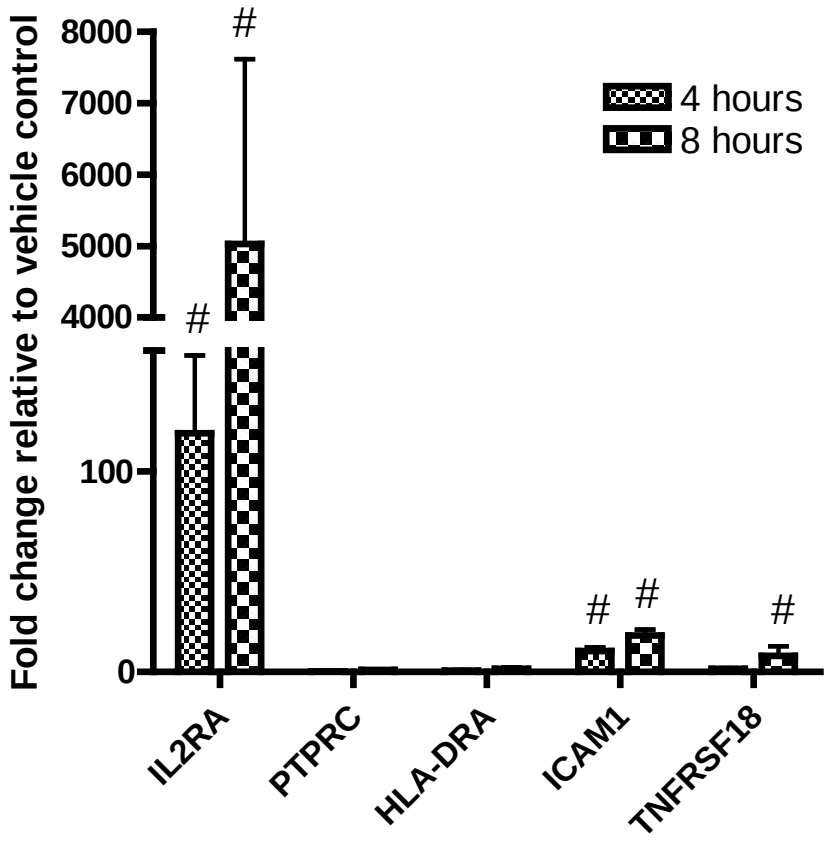


Figure 4.15 Transcriptional profile of genes encoding adhesion molecules and cell surface proteins in primary human macrophages following M. oryzae stimulation. *Primary human macrophages were stimulated with M. oryzae at a multiplicity of infection of 200 for four and eight hours. Each column represents the mean ± standard error of three experiments. The fold change of each transcript was generated by comparing the means of signal densities of stimulated vs non-stimulated control. Fold change >2 (#).*

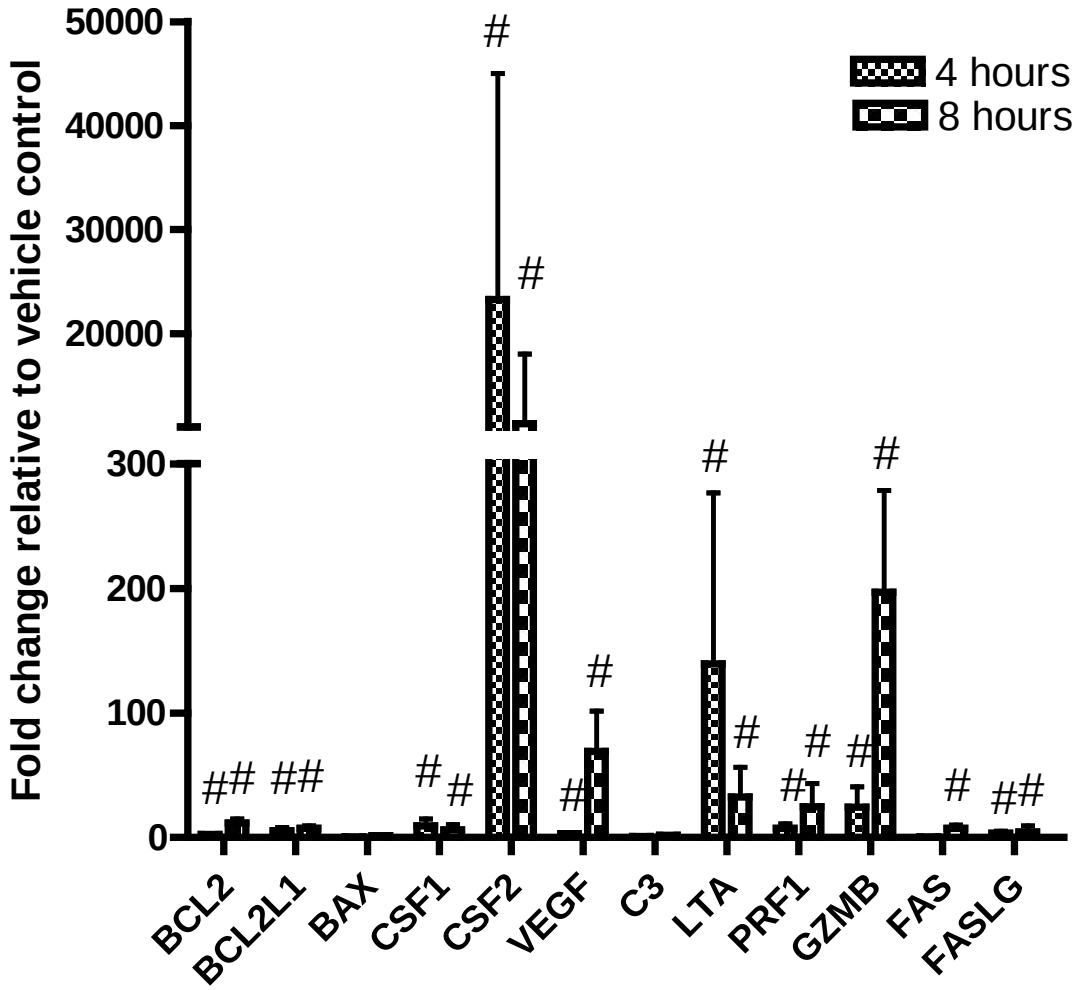


Figure 4.16 Transcriptional profile of genes encoding growth factors, immune effectors and pro-survival proteins in primary human macrophages following M. oryzae stimulation.

Primary human macrophages were stimulated with M. oryzae at a multiplicity of infection of 200 for four and eight hours. Each column represents the mean ± standard error of three experiments. The fold change of each transcript was generated by comparing the means of signal densities of stimulated vs non-stimulated control. Fold change >2 (#).

4.2.10.3 Biological pathway analysis by Ingenuity IPA analysis of human primary macrophages infected with *M. oryzae*

Genes identified as being differentially expressed in primary human macrophages following *M. oryzae* were analysed using IPA pathway analysis software (Ingenuity System Inc, USA). Analysis was used to interpret the eight hour data and conditions were matched to those used in *section 4.8* with the exception that human instead of murine pathways were cross-referenced.

IPA analysis revealed significant biological pathways and canonical pathways associated with the pattern of differential gene expression following *M. oryzae* infection. The transcriptional profile observed in the infected human primary macrophages correlated with expression seen during 'immunological disease' (p=2.93E-32 to 1.55E-15) and 'inflammatory disease' (p=8.90E-27 to 2.21E-15). Similar to our previous study in murine cell lines (*Section 4.8*) we observed that the transcription profile of human infected macrophages was significantly related to cardiotoxicity canonical pathways. Both 'cardiac inflammation' (p=8.76E-11 to 1.51E-02) and 'cardiac necrosis/cell death' (p=2.4E-14 to 4.7E-02) were significantly related to the transcription profile identified following *M. oryzae* infection.

4.2.11 TLR response to *M. oryzae*

In order to determine whether *M. oryzae* could stimulate TLR signalling, TLR deficient HEK-293 cells were transfected with a nuclear factor-kappaB (NF-*k*B)-sensitive reporter and TLR2 or TLR4 (kindly performed by Dr Clett Erridge, University of Leicester). All TLR signalling pathways culminate in activation of the transcription factor nuclear factor-kappaB (NF-*k*B), which controls the expression of an array of inflammatory cytokine genes. Previous studies have demonstrated that TLR2 and TLR4 are the most abundant TLRs in human atherosclerotic lesions and that transcription was elevated >3-fold compared to healthy arteries (Edfeldt et al., 2002). With this in mind, the following work concentrates on these two receptors. TLR2 mediates host response to bacteria principally by recognition of the lipopeptides (e.g. Lipoteichoic acid), and TLR4 mediates host response to bacteria by recognition of lipopolysaccahride (LPS). *E. coli* is known to stimulate both TLR2 and TLR4 (Lee et al., 2002).

M. oryzae and *E. coli* K12 (starting concentration of $1x10^8$ cells/ml), Pam3CSK4 (10ng/ml) (a TLR2 agonist) and LPS (10ng/ml) were suspended in DMEM and incubated with transfected HEK-293 cells for 18 hours. NF-*k*B signalling was expressed as mean fluorescent intensity of the reporter construct (pELAM) normalised to co-transfected renilla expression to account for fluctuations in plasmid transfection and expression efficiency. Clear TLR2 and TLR4 dependent signalling of Pam3CSK4 and LPS, respectively were detected (*figure 4.17* and *figure 4.18*). As expected, *E. coli* activated signalling via both TLR2 and TLR4. It was also observed that LPS was a mild activator of TLR2-mediated signalling.

TLR4 was entirely insensitive to *M. oryzae* (*figure 4.18*). Mild TLR2 dependent signalling was observed when TLR2 HEK-293 transfected cells were challenged with the highest concentration of *M. oryzae* ($1x10^7$ cells/ml) (*figure 4.17*). However at decreasing concentrations ($1x10^6$ and $1x10^5$ cells/ml) no significant increase in TLR2 signalling relative to the control was observed. Signalling detected in the negative controls represents homeostatic NF-*k*B signalling. All experiments were carried out in triplicate. These data suggest that *M. oryzae* expresses small amounts of TLR2 ligand but does not possess the TLR4 ligand LPS.

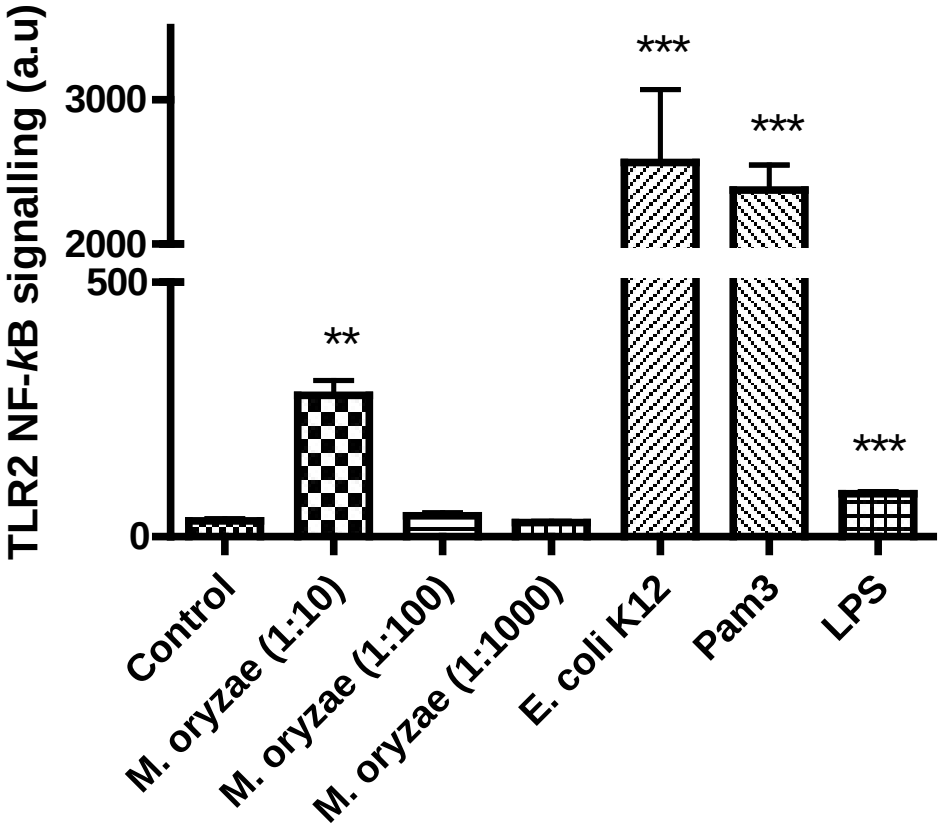


Figure 4.17 M. oryzae and TLR2 signalling.

HEK-293 cells co-transfected with NF-kB sensitive reporter (pELAM) and TLR2 were challenged for 18 hours with M. oryzae (1:10 dilution equivalent to $1x10^7$ cells/ml), E. coli K12 ($1x10^7$ cells/ml), Pam3CSK4 (10ng/ml) and LPS (10ng/ml). The vehicle control was treated with medium only. Arbitrary units (a.u.) represents mean fluorescent intensity of the reporter construct normalised to co-transfected renilla. Data are representative of three experiments ± S.D. $^{**}p<0.01$ vs. control; $^{***}p<0.001$ vs. control.

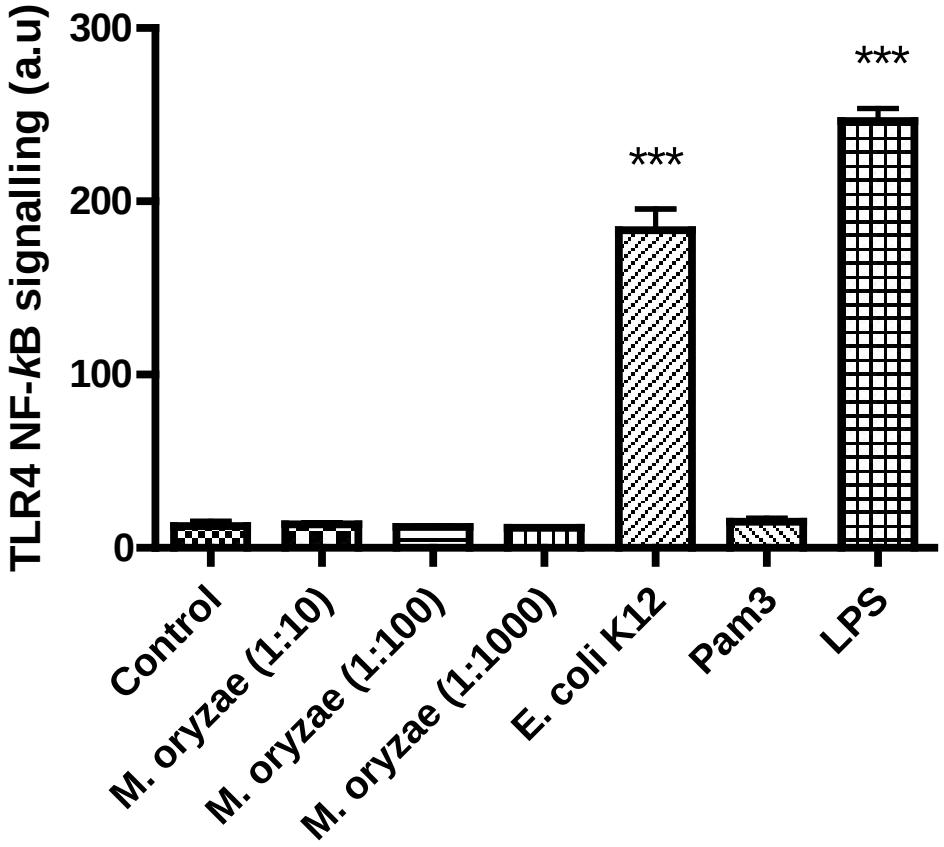


Figure 4.18 M. oryzae and TLR4 signalling.

HEK-293 cells co-transfected with NF-kB sensitive reporter (pELAM) and TLR4 were challenged for 18 hours with M. oryzae (1:10 dilution equivalent to $1x10^7$ cells/ml), E. coli K12 ($1x10^7$ cells/ml), Pam3CSK4 (10ng/ml) and LPS (10ng/ml). The vehicle control was treated with medium only. Arbitrary units (a.u.) represents mean fluorescent intensity of the reporter construct normalised to co-transfected renilla. Data are representative of three experiments ± S.D. $^{**}p<0.01$ vs. control; $^{***}p<0.001$ vs. control.

4.2.12 Detection of LPS in *M. oryzae*

In *section 4.11,* it was determined that *M. oryzae* does not stimulate TLR4 signalling. To determine whether *M. oryzae* possesses a TLR4 ligand, total cellular LPS and LOS was isolated from the bacteria using a hot phenol/water extraction protocol. To ensure extraction efficacy, LPS from *E. coli* K12 was extracted in tandem. In order to characterise extracted LPS/LOS, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining was performed. Commercially available (Sigma-Aldrich) *Salmonella typhimurium* rough LPS, *Salmonella typhimurium* smooth LPS, Pam3CSK4 and LOS were also visualised for comparison.

LPS profiling discriminated between isolated *E. coli* LPS, *Salmonella* rough LPS, *Salmonella* smooth LPS and LOS (*figure 4.19*). The LPS extracted from *E. coli* exhibited similar band patterns to *Salmonella* smooth LPS. *Salmonella* rough LPS was characterised by having no repeating units. SDS-PAGE confirmed the absence of the TLR4 ligands LPS and LOS in *M. oryzae*.

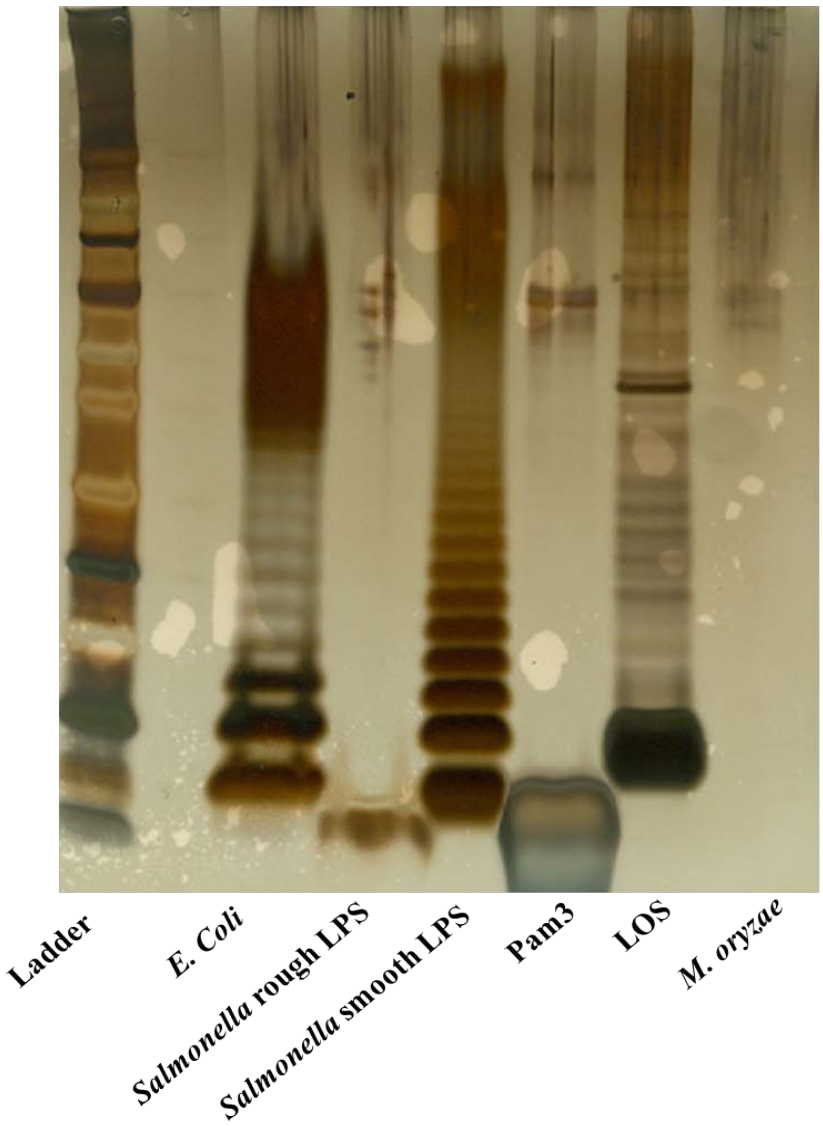


Figure 4.19 LPS/LOS profile of M. oryzae

SDS-PAGE (13%) and silver staining of LPS/LOS following hot phenol extraction from M. oryzae.

E. coli K12 was used as the positive control. Smooth and rough LPS from Salmonella typhimurium and purified LOS were included for visual comparison (5µg per lane).

4.3 Discussion

Bacterial DNA from a diverse range of species was identified in biopsies of aortic adventitial tissue obtained from patients following CABG surgery. The principle finding of this study was that RA patients had a significantly reduced heterogeneity in the bacterial species associated with the diseased aortic adventitia when compared to non-RA patients. Furthermore, *M. oryzae* was detected in all aortic adventitia samples from RA patients that were PCR-positive for the 16S rRNA gene, but in only 25% of samples from non-RA patients. The clinical relevance of *M. oryzae* is further discussed in *chapter 6* (general discussion).

The bacteria identified in this study included an array of environmental and oral species. Many of these species have previously been reported as opportunistic or nosocomial pathogens. For example *Stenotrophomonas* sp., which was detected in both RA and non-RA patients, has recently emerged as an important opportunistic pathogen in debilitated individuals (Looney et al., 2009). Furthermore, it is reported to infect immunocompromised individuals at a high frequency (Rolston et al., 2005). Indeed, sequences of *Stenotrophomonas* sp., have been reported in up to 60% of aneurysm samples (Marques da Silva et al., 2006). *Stenotrophomonas* sp., have also been demonstrated to cause bloodstream infections (Park et al., 2008). Several species of *Stenotrophomonas,* including the most widely studied *S. maltophilia,* express a protease (*StmPr1*) that is capable of breaking down fibrinogen, fibronectin and collagen (Windhorst et al., 2002). This may ultimately result in local tissue damage and as such, may be a potential atherogenic trigger.

Several oral bacteria were detected in the present study, including *Veillonella* sp., *Porphyromonas endodontalis, Streptococcus sanguinis. Streptococcus mitis*, *Haemophilus* and a *Neisseria* oral clone. *Veillonella* species exist as part of a healthy oral, gastrointestinal and female genital flora. However, in a rare number of cases *Veillonella* species have been implicated as the aetiological agent of infective endocarditis (Greaves and Kaiser, 1984, Loughrey and Chew, 1990, Loewe et al., 1946), a condition that shares many pathophysiological features with atherosclerosis. Similarly, *Streptococcus sanguinis* has been reported to be a causative agent in endocarditis (Douglas et al., 1993). The detection of oral bacteria in the aortic adventitia provides support for the concept of

haematogenous spread of oral pathogens to the atherosclerotic site. Oral bacteria can potentially enter the bloodstream during tooth brushing, chewing, oral infection and invasive dental procedures (Chung et al., 1986).

Surprisingly, despite a variety of oral pathogens having been identified in the aortic adventitia, not a single periodontal pathogens was detected in these samples. Periodontal disease has been proposed as a significant risk factor in CVD (Destefano et al., 1993, Mattila et al., 1989). In contrast to data presented in this study, Haraszthy *et al*, reported that 22 of 50 endarterectomy samples tested positive for the presence of periodontal pathogens (Haraszthy et al., 2000).

Chlamydia pneumoniae has been the subject of the majority of research investigating bacterial infection and atherosclerosis. Strong associations between *C. pneumoniae* and atherosclerosis have been demonstrated by: (i) detection of *C. pneumoniae* within atherosclerotic lesions, but not adjacent normal tissue by PCR, IHC and electron microscopy (Shor et al., 1992, Kuo et al., 1993); (ii) sero-epidemiological studies demonstrating that patients with CVD have higher titres of anti-*C. pneumoniae* antibodies compared to healthy patients; (iii) showing that *C. pneumoniae* can cause exacerbation of atherosclerotic lesions in murine models (Hu et al., 1999, Campbell et al., 2000). Nonetheless, the association between *C. pneumoniae* and CVD has been inconsistent from study to study, in a manner that has been ascribed to varying diagnostic methodology (Boman and Hammerschlag, 2002). However, as no *C. pneumoniae* was detected in any of the samples studied here, it would appear unlikely that *C. pneumoniae* plays a major role in adventitial derived pathology.

The differences in bacterial species identified in this study compared with other studies could have several explanations. These include differences in geographical location of patients sampled, diagnostic methodology used and anatomical site sampled.

Clinical samples used for the present study were all taken from an age- and sex-matched Norwegian cohort. Owing to socioeconomic status, diet, environment and lifestyle the microbial profiles are likely to vary from one geographic location to another. Research to determine the geographical variation in aortic microbiota remains to be performed. However, Haffajee *et al*, determined that the microbial profile of subgingival plaque

samples from chronic periodontitis subjects varied significantly across four analysed countries and these differences persisted despite adjustment for age, gender and smoking status (Haffajee et al., 2004).

PCR-based sequence analysis of the 16S rRNA gene was used to identify bacterial DNA in the aortic adventitia. 16S rRNA gene analysis is a powerful and essential tool for studying bacterial diversity and has enabled detection of unculturable bacteria. However, the PCR procedure intrinsically leads to bias and error. This is attributable to complex factors such as preferential annealing between primers and templates, self-annealing between primers and templates, self-annealing between PCR products, different copy numbers of the targeted genes and the formation of chimeric sequences (Suzuki and Giovannoni, 1996, Qiu et al., 2001, Hongoh et al., 2003). In the present study, a low annealing temperature of 55^{o}C was used so as to suppress amplification bias caused by primer mismatches, and the number of PCR cycles was limited to 30 in an effort to minimise primer mismatch but still retain detection sensitivity. Despite the widespread use of 16S rRNA gene PCR in clinical research, there remains no conformity with regard to either primer design or PCR conditions. These anomalies are likely to contribute to the variations observed in data generated from different research groups.

Another major concern when using 16S rRNA gene sequencing analysis to identify bacteria in clinical specimens is the lysis efficiency of different bacteria in a complex environment. Ideally, lysis of bacterial cells should be equally efficient for all bacterial species and independent of growth phase and the concentration of cells in the sample (Zoetendal et al., 2001). The present study employed a powerful DNA isolation mechanism involving proteinase K digestion prior to homogenisation in Trizol® reagent. This technique differs from other published work studying 16S rRNA gene presence in human tissue and may lead to differences in the bacterial species detected. For example, Marques da Silva *et al*, (2006) homogenised tissue samples in a sterile mortar with liquid nitrogen whereas Haraszthy *et al* (2000) purified DNA directly from tissue samples.

Importantly, the present study is unique as it was carried out in aortic adventitia tissue that was removed for the establishment of aortocoronary anastomoses during CABG surgery. This tissue is located downstream of the site of atherosclerotic lesion. Considerable attention has been given to the atherosclerotic lesion itself, as well as endothelial cells on

the luminal surface of arteries and the media with regards infection and vascular lesion formation. However, virtually no attention has been paid to the potential role of the adventitia in this process. This lack of research attention may be due to the previous perception that the adventitia is nothing more than 'loose connective tissue' that enclosed blood vessels and is devoid of function other than to act as support for large vessels (Wilcox and Scott, 1996). Contrary to this, in chapter 3 of this thesis it was determined that the adventitia is a site of considerable immunological activity, possessing inflammatory infiltrate and detectable levels of TNFα, heat shock protein and calprotectin. Furthermore, the adventitial layer of vessels have been suggested to exhibit properties resembling a stem/progenitor cell niche. Indeed, progenitor cells have been isolated from the adventitia of both human and murine blood vessels with the potential to form endothelial, osteogenic and mural cells (Majesky et al., 2012). Progenitor cells from the adventitia have been suggested to migrate into the intima where they proliferate and differentiate into neo-smooth muscle cells which play a functional role in the formation of the atherosclerotic lesion (Xu, 2005).

Adventitia-derived inflammatory cells, cytokines, progenitor cells and microvasculature likely contribute significantly to many different vascular disease states (Maiellaro and Taylor, 2007). The involvement of the adventitia in vascular pathology suggests that concomitant to the traditional 'inside-out' view of atherogenesis that there is an 'outside-in' component. The establishment of bacterial presence in this under-studied niche suggests that bacterial infection may trigger an 'outside-in' inflammatory response. However, future research is required to verify this hypothesis.

The detection of bacterial DNA in the aortic adventitia supports the hypothesis that bacterial infection may contribute to the pathophysiology of atherosclerosis, but to what extent remains uncertain. It has been postulated that the atherosclerotic tissue may act as a 'mechanical sieve' trapping bacteria present in blood circulation and that detected bacteria, although present, may have no pathological significance (Lehtiniemi et al., 2005). Furthermore, it is possible that bacterial colonisation is secondary to atheromatous lesion formation and may potentiate acceleration of the disease process. Extending our understanding of the principal factors that lead to accelerated atherogenesis in RA patients is necessary in order for specific therapeutic intervention and prevention to be implemented.

The findings of our study implicate a diverse range of bacterial species within the atherosclerotic lesion, supporting the hypothesis that a wide range of bacterial species rather than a single aetiological agent may contribute to atherosclerotic burden. In keeping with this, Ott *et al*. (Ott et al., 2006) reported a mean bacterial diversity of 12.33 different species in 38 CHD patients studied. Furthermore, the current study has demonstrated that the bacterial population in the aortic adventitia of CABG patients is significantly less diverse in patients with RA (see *general discussion*). These data may help in the on-going debate to explain why the incidence of CVD is significantly elevated in RA patients.

5 Systemic immune profile in cardiovascular disease with co-existing rheumatoid arthritis and implications in foam cell formation

5.1 Introduction

The reasons for the increased CVD morbidity and mortality observed in RA are likely to be multifactorial, with the majority of data supporting a predominant role for systemic inflammation in driving premature atherogenesis.

Evidence suggests that atherosclerosis is an inflammatory disease sharing a similar pathophysiology to synovial inflammation and pannus formation in RA (Snow and Mikuls, 2005). Furthermore, these diseases share several pathogenic features including the predominant role of pro-inflammatory cytokines (e.g. TNFα and IL-6), angiogenesis, increased expression of leukocyte adhesion molecules and the elevation of serum acute phase proteins (e.g. CRP and fibrinogen).

Population-based studies support an association of cardiovascular events with inflammation. In a study involving over 100 male participants, base-line plasma CRP was identified as an independent predictor for future myocardial infarction (Ridker, 1997, Ridker et al., 2002). The men in the quartile with the highest CRP had three times the risk of myocardial infarction compared to men in the lowest quartile. In a joint statement the Centre for Disease Control (CDC) and the American Heart Association (AHA) categorised individuals with serum levels of CRP >3mg/L as high risk for future CVD events (Graf et al., 2009). Serum CRP levels in RA patients are frequently above the 3mg/L cut-off associated with high risk of CVD in the general population. For example, cross-sectional examination of 767 RA patients revealed a median CRP serum level of 11mg/L (Aletaha et al., 2005).

Endothelial dysfunction as a result of reduced vasodilator function is among the earliest features of atherosclerosis (Gotto, 2004). Systemic inflammation observed in RA may act as a potent mediator of endothelial dysfunction. For example, *in vivo* human studies demonstrate that pro-inflammatory cytokines induce transient and reversible endothelial dysfunction (Bhagat and Vallance, 1997), an effect that may be mediated by increased oxidative stress and reduced vascular nitric oxide bioavailability (Clapp et al., 2004).

The notion that systemic inflammation present in RA alters vasodilation was reinforced in a study involving 32 RA patients under the age of 60. Young to middle aged RA patients with low disease activity without clinically overt atherosclerosis or traditional CVD risk patterns were found to have significant reductions in brachial flow-mediated vasodilation and these reductions positively correlated with serum CRP levels (Vaudo et al., 2004).

If the hypothesis that inflammation as a result of RA contributes to the development of CVD events is correct, then it is probable that the use of anti-inflammatory therapeutics in RA may decrease CVD morbidity. Consistent with this, Jacobsson *et al*., examined 983 RA patients, 531 of which were receiving treatment with the anti-TNF agents etanercept or infliximab and observed that subsequent CVD events were considerably lower in the anti-TNF-treated patients (Jacobsson et al., 2005). Halm *et al.*, investigated the association between CVD and the use of conventional disease-modifying antirheumatic drugs (DMARDs) in 613 RA patients. After correction for other CVD risk factors DMARD use was associated with a significant CVD risk reduction. The risk reductions remained significant after additional correction for the presence of rheumatoid factor and erosions (van Halm et al., 2006). These studies highlight the protective role of anti-inflammatory therapeutics for the risk of CVD morbidity in RA patients

Macrophages play a central role in atherosclerosis as modulators of both the immune response and lipid metabolism. A critical event in atherogenesis is the focal accumulation of foam cells derived from macrophages (Shashkin et al., 2005). Foam cell formation is thought to be induced by Low Density Lipoproteins (LDL), including acetylated LDL (acLDL) and oxidised LDL (oxLDL). Although far less well established, it has also been suggested that the microvasculature has a pathogenic role in RA (Rothschild and Masi, 1982) and immunohistochemical analysis has identified foam-like cells within the region of synovial blood vessels (Winyard et al., 1993).

Scavenger receptors are known to be involved in the transformation of macrophages into lipid-laden foam cells (de Villiers and Smart, 1999). The term “scavenger receptor” initially described the macrophage cell surface proteins that mediate the uptake of modified LDL in cell culture (Goldstein et al., 1979). Eight classes of macrophage scavenger receptors exist (Classes A to H). Among the growing number of scavenger receptor members, SR-A1, SR-B1, MARCO and CD36 which recognise both oxLDL and acLDL are thought to be the principal receptors responsible for the uptake of modified LDL and the subsequent formation of foam cells (Kunjathoor et al., 2002, McLaren et al., 2010).

The uptake of oxLDL and acLDL, the primary modified LDL particles found in atherosclerotic plaque, is increased in THP-1 macrophages following stimulation with IFN-γ (Wuttge et al., 2004, Reiss et al., 2004). Furthermore, IFN-γ was found to increase SR-A expression in early differentiated THP-1 macrophages (Grewal et al., 2001).

Macrophages are inherently reactive and respond to a broad range of cytokines including IL-4, IL-13 and TNFα (Stein et al., 1992, Doherty et al., 1993, Khera et al., 2011).

It thus seems probable that pro-inflammatory factors that are over expressed in the serum of patients with RA+CVD may prime macrophages to enhance the uptake of modified lipoprotein resulting in increased foam cell formation.

To increase understanding of the link between RA and CVD we collected human serum samples from healthy controls as well as patients with RA, CVD and RA+CVD. The principal aims of this study were:

1. To determine which pro-inflammatory markers and immune mediators are over-expressed in patients with RA+CVD relative to healthy controls and patients with RA or CVD only.

2. To uncover whether inflammatory markers over-expressed in RA+CVD may have implications in modified LDL uptake and subsequent foam cell formation.

5.2 Results

5.2.1 Patients characteristics

Human serum samples were from the Feiring Heart Biopsy Study. Patient characteristics are shown in *Table 5.1*. A total of 77 patients were included in the following human studies, and comprised patients with CVD (n=19), RA (n=21), RA+CVD (n=16) and healthy controls (n=21). Attempts were made to minimise inter-cohort variability of both the demographic data and pre-existing cardiovascular risk factors. However, owing to the inherent differences in the diseases under investigation, and the limited samples available, this was not always possible.

There was no significant difference in the gender composition of each cohort, however the mean age of the healthy group was significantly lower than both the CVD and RA+CVD patients. As expected, the incidence of hypertension and hyperlipidaemia was significantly elevated in the CVD and RA+CVD cohorts. There was a significant increase in the number of current smokers in the RA cohort relative to both the RA+CVD and CVD cohorts. Data regarding both current and previous smoking was unavailable for the healthy controls.

The use of current cardiovascular medication (ACE inhibitors, acetylsalicyclic acid and statins) was similar in both the CVD and RA+CVD cohorts. Most of the patients with RA exhibited low to moderate disease activity. The use of DMARDs was similar in both the RA and RA+CVD cohorts; however the use of traditional non-steroidal anti-inflammatory drugs (NSAIDs) was greater in the RA cohort relative to the RA+CVD cohort. This anomaly is accounted for by the significantly increased use of COX-2 selective inhibitors, which are a form of NSAID, in the RA+CVD cohort relative to the CVD cohort.

	CVD (n=19)	RA (n=21)	RA+CVD (n=16)	Healthy (n=21)	*P* †
Age-years	68±9	62±8	69±9	60±9	<0.05‡
B.M.I.	25±4	25±5	26±6	NA	0.65
Female-no. (%)	5 (26)	14 (66)	6 (38)	10 (48)	0.07
Duration of CVD - months	88±80	-	97±110	-	0.9
Time from angiography to CABG – days	32±61	-	20±32	-	0.49
Duration of RA - months	-	126±114	221±184	-	0.14
Number of swollen joints	-	9±10	4±6	-	0.12
C- reactive protein - mg/l	3±3	7±9	11±10	1±0.4	<0.0001§
Erythrocyte sedimentation rate - mm/hour	15±9	19±17	34±28	4±3	<0.0001ϕ
Hypertension - no. (%)	8 (42)	6 (29)	10 (62)	0 (0)	<0.001¥
Hyperlipidemia - no. (%)	19 (100)	2 (10)	13 (81)	0 (0)	<0.0001Ж
History of myocardial infarction - no (%)	8 (42)	0 (0)	11 (69)	0 (0)	<0.0001₳
Diabetes - no (%)	1 (5)	0 (0)	1 (6)	1 (5)	0.74
Acute coronary syndrome - no (%)	2 (11)	0 (0)	4 (25)	0 (0)	<0.05₱
Previous smoker - no (%)	11 (58)	0 (0)	6 (37)	NA	<0.001α
Current smoker - no (%)	1 (5)	11 (52)	2 (12)	NA	<0.05Ƶ
Current use of:					
Disease modifying drugs - no. (%)	0 (0)	18 (86)	14 (88)	0 (0)	<0.0001₳
Ace inhibitors - no. (%)	6 (32)	1 (5)	4 (25)	0 (0)	<0.05Ψ
Traditional NSAIDs - no. (%)	0 (0)	7 (33)	2 (12)	0 (0)	<0.0001Ł
Cox2-selective inhibitors - no. (%)	0 (0)	0 (0)	4 (25)	0 (0)	<0.05♯
Traditional statins - no. (%)	18 (95)	2 (10)	13 (81)	0 (0)	<0.0001₡
Acetylsalicylic acid - no. (%)	16 (84)	1 (5)	14 (87)	0 (0)	<0.0001₴
Patient global assessment of RA (VAS, 0-100mm)	-	4±2	3±2	-	0.053

Table 5.1 Patient characteristics

Unless indicated otherwise, values represent mean ± SD. Numbers may not add up to the expected total due to missing data for some variables. ACE: angiotensin-converting enzyme. NSAIDs: non-steroidal anti-inflammatory drugs. VAS: visual analogue scale. NA: Not available

† *P* values for continuous dependent variables are from Kruskal-Wallis test followed by Dunn's multiple comparison test. Where only two cohorts have available data, analysis is by Mann-Whitney U test

‡ *P<0.05 for comparisons between healthy and CVD or RA+CVD*

§ *P<0.05 for comparisons between healthy and CVD. P<0.01 for comparisons between healthy and RA. P<0.001 for comparisons between healthy and RA+CVD.*

ϕ *P<0.01 for comparisons between healthy and CVD, RA or RA+CVD*

¥ *P<0.05 for comparisons between healthy and CVD or RA+CVD*

Ж *P<0.001 for comparisons between: healthy and CVD or RA+CVD; CVD and RA; RA and RA+CVD*

Ⱥ *P<0.05 for comparisons between CVD and healthy or RA. P<0.001 for comparisons between RA+CVD and healthy or RA*

₱ *P<0.05 for comparisons between RA+CVD and healthy or RA*

α *P<0.05 for comparisons between RA and RA+CVD. P<0.001 for comparisons between CVD and RA*

Ⱦ *P<0.05 for comparisons between RA and CVD or RA+CVD*

Ⱥ *P<0.001 for comparisons between: healthy and RA or RA+CVD; CVD and RA or RA+CVD*

Ψ *P<0.05 for comparisons between healthy and CVD*

Ł *P<0.05 for comparisons between RA and RA+CVD. P<0.001 for comparisons between RA and healthy or CVD*

♯ *P<0.01 for comparisons between RA+CVD and healthy, RA, or CVD*

₵, ₴ *P<0.001 for comparisons between: CVD and healthy or RA; RA+CVD and healthy or RA*

5.2.2 Immune expression in serum of CVD, RA and RA+CVD patients

In order to gain a better understanding of systemic inflammatory-derived events occurring within RA and CVD, a broad range of cytokines, chemokines, growth factors and receptors were analysed in RA (n=21), CVD (n=19) and RA+CVD patients (n=16) as well as healthy controls (n=21) (*see section 5.2.1*). Protein concentrations within human serum samples were measured by LUMINEX and ELISA (sST2 only). Details of the proteins analysed are outlined in *table 5.2 and table 5.3*.

The research outlined in the following sections concentrates on uncovering potential biological mediators that may contribute to the increased CVD burden observed in RA patients. Particular attention was paid to biomarkers that were significantly elevated in RA+CVD or RA patients relative to CVD patients or healthy controls. Elevation of biomarkers in these patients could potentially lead to loss of vascular homeostasis and potentiate the onset of atherosclerosis.

Protein	Name	Class	Functions
IL-1β	Interleukin 1 β	Cytokine	Initiates cytokine cascade and vascular adhesion expression
IL-2	Interleukin 2	Cytokine	Stimulates proliferation of T and B cells
IL-4	Interleukin 4	Cytokine	Induces differentiation of naive helper T cells to Th2 cells
IL-5	Interleukin 5	Cytokine	Stimulates B cell growth and mediates eosinophil activation
IL-6	Interleukin 6	Cytokine	Pro-inflammatory pleiotropic cytokine
IL-10	Interleukin 10	Cytokine	Anti-inflammatory/resolving
IL-12	Interleukin 12	Cytokine	Differentiation of Th1 and Th2 cells and stimulates IFNγ production by PBMCs
IL-13	Interleukin 13	Cytokine	Inhibits inflammatory cytokine production
IL-15	Interleukin 15	Cytokine	Stimulates proliferation of T-lymphocytes
IL-17	Interleukin 17	Cytokine	Induces stromal cells to produce proinflammatory and hematopoietic cytokines
TNFα	Tumour necrosis factor alpha	Cytokine	Regulation of a wide spectrum of biological processes including cell proliferation, differentiation, lipid metabolism and apoptosis
IFNα	Interferon α	Cytokine	Produced by macrophages and have antiviral activities
GM-CSF	Granulocyte macrophage colony stimulating factor	Cytokine	Stimulates growth and differentiation of hematopoietic precursor cells from various lineages, including macrophages, eosinophils, granulocytes and erythrocytes
MIG	Gamma interferon induced monokine	Cytokine	Chemotactic for activated T cells
Eotaxin	Eotaxin	Cytokine	Recruits eosinophils
G-CSF	Granulocyte colony stimulating factor	Cytokine	Controls the production, differentiation and function of granulocytes
IL-1 RA	Interleukin 1 receptor antagonist	Cytokine	Natural inhibitor of the pro-inflammatory effect of IL-1α and IL-1β

Table 5.2 List of cytokines examined in the serum of healthy, RA, CVD and RA+CVD patients

Protein	Name	Class	Functions
FGF	Fibroblast growth factor	Growth factor	Regulates cell growth, morphogenesis and tissue repair
HGF	Hepatocyte growth factor	Growth factor	Acts as a growth factor for a broad spectrum of tissues and cell types
IL-7	Interleukin 7	Growth factor	Stimulates proliferation of lymphoid progenitors and regulates B cell maturation
VEGF	Vascular endothelial growth factor	Growth factor	Induces endothelial proliferation and migration. Inhibits apoptosis and induces permeabilisation of blood vessels
EGF	Epidermal growth factor	Growth factor	Stimulates growth of various epidermal and epithelial tissue
IL-2R	Interleukin 2 receptor	Receptor	High affinity receptor for IL-2
sST2	Soluble suppression of tumorugenicity 2	Receptor	May be involved in the function of T helper cells. Receptor for Il-33 which induces T cells, mast cells and basophils to produce type 2 cytokines
IL-8	Interleukin 8	Chemokine	Poly morphonuclear neutrophil chemoattractant
MIP1α	Macrophage inflammatory protein 1α	Chemokine	Involved in the acute inflammatory state for the recruitment and activation of polymorphonuclear leukocytes
MIP1β	Macrophage inflammatory protein 1β	Chemokine	Attracts $CD4^+$ T lymphocytes
IP-10	IP-10	Chemokine	Chemotactic effect on activated T, NK and TH-1 cells
RANTES	Rantes	Chemokine	Promotion of inflammatory infiltrate and T-cell chemotaxis
MCP-1	Monocyte chemotactic protein 1	Chemokine	Chemotactic factor that attracts monocytes and basophils but not neutrophils or eosinophils

Table 5.3 List of growth factors, receptors and chemokines examined in the serum of healthy, RA, CVD and RA+CVD patients

5.2.2.1 Cytokine profile in CVD, RA, RA+CVD subjects

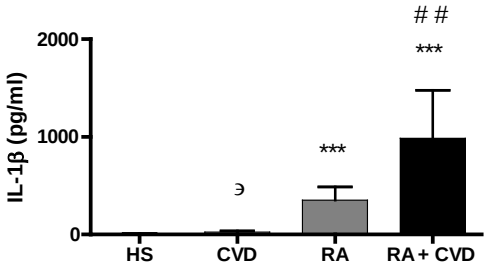
The serum level of 17 different cytokines (*table 5.2*) was investigated in CVD (n=19), RA (n=21) and RA+CVD (n=16) patients and healthy controls (n=21) by Luminex assay. Mean cytokine levels in pg/ml in whole serum shown in *figure 5.1-5.2.*

The expression of all cytokines, with the exception of G-CSF was significantly elevated in the RA+CVD cohort relative to the healthy controls (*Figure 5.1 & Figure 5.2*). When considering RA patients relative to healthy controls, 12 of the 17 cytokines examined were elevated, four remained unchanged (IL-5, IL-10, IL-13, IL-17), and G-CSF was significantly reduced.

Somewhat surprisingly, IL-17 was found to be significantly elevated in the RA+CVD cohort relative to the RA cohort. The T cell cytokine IL-17 is known to promote inflammation in RA through induction of monocyte migration (Shahrara et al., 2009). Il-17 was not significantly increased in CVD patients relative to healthy controls. Additionally, there was a non-significant trend towards elevated IL-1β, IL-2, IL-5 and IL-13 expression in the RA+CVD cohort relative to the RA cohort.

Although CVD is an inflammatory condition, there was an overwhelming trend for reduced cytokine expression in CVD patients compared to RA patients. IL-1β, IL-2 and IL-6 were significantly lower in CVD patients compared to RA patients, and there was a non-significant trend towards reduced expression in all but one (G-CSF) of the remaining cytokines under investigation. Furthermore, IL-12 was the only cytokine under investigation to show significant elevation in the serum of CVD patients relative to healthy controls.

The pattern of G-CSF expression was distinct from all other cytokines analysed in this study. Healthy controls and RA+CVD patients had high levels of serum G-CSF (875±363 and 1832±837, respectively) where as both CVD and RA patients expressed ~10-fold less (105±5 and 66±8, respectively).


IL-1β (pg/ml)
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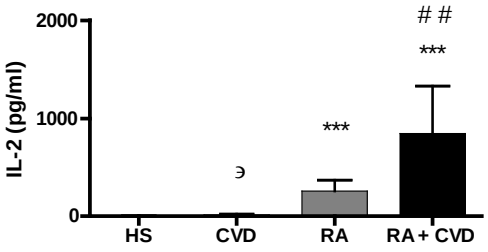
HS
CVD
RA
RA + CVD


IL-2 (pg/ml)
2000
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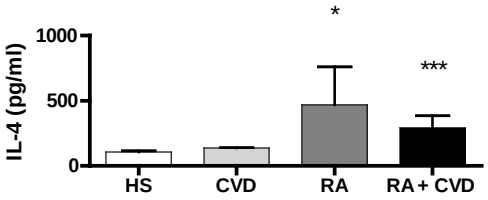
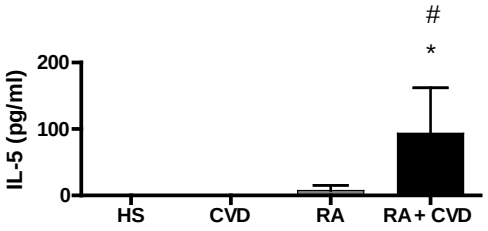
HS
CVD
RA
RA + CVD


IL-4 (pg/ml)
1000
500
0
*
***
HS
CVD
RA
RA + CVD


IL-5 (pg/ml)
200
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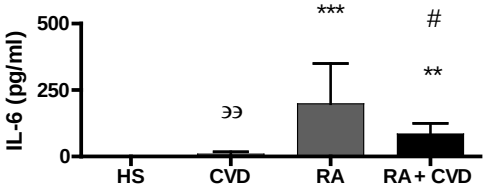
*
HS
CVD
RA
RA + CVD


IL-6 (pg/ml)
500
250
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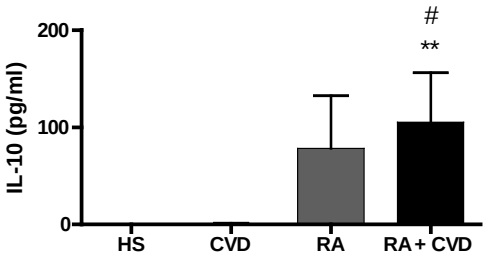
ƏƏ
HS
CVD
RA
RA + CVD


IL-10 (pg/ml)
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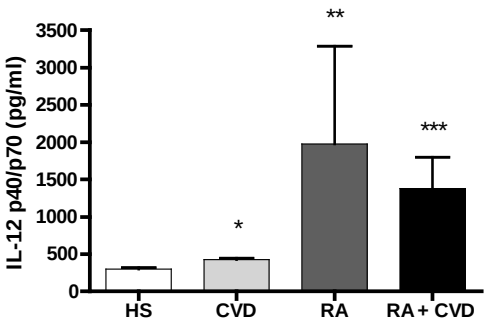
HS
CVD
RA
RA + CVD


IL-12 p40/p70 (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
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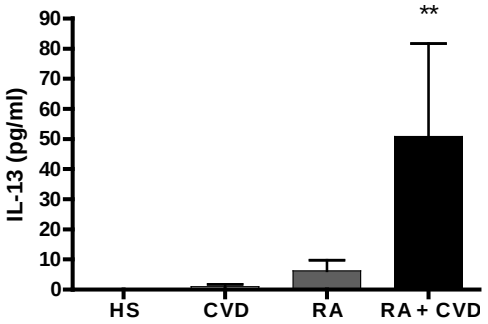
*
HS
CVD
RA
RA + CVD


IL-13 (pg/ml)
90
80
70
60
50
40
30
20
10
0
**
HS
CVD
RA
RA + CVD

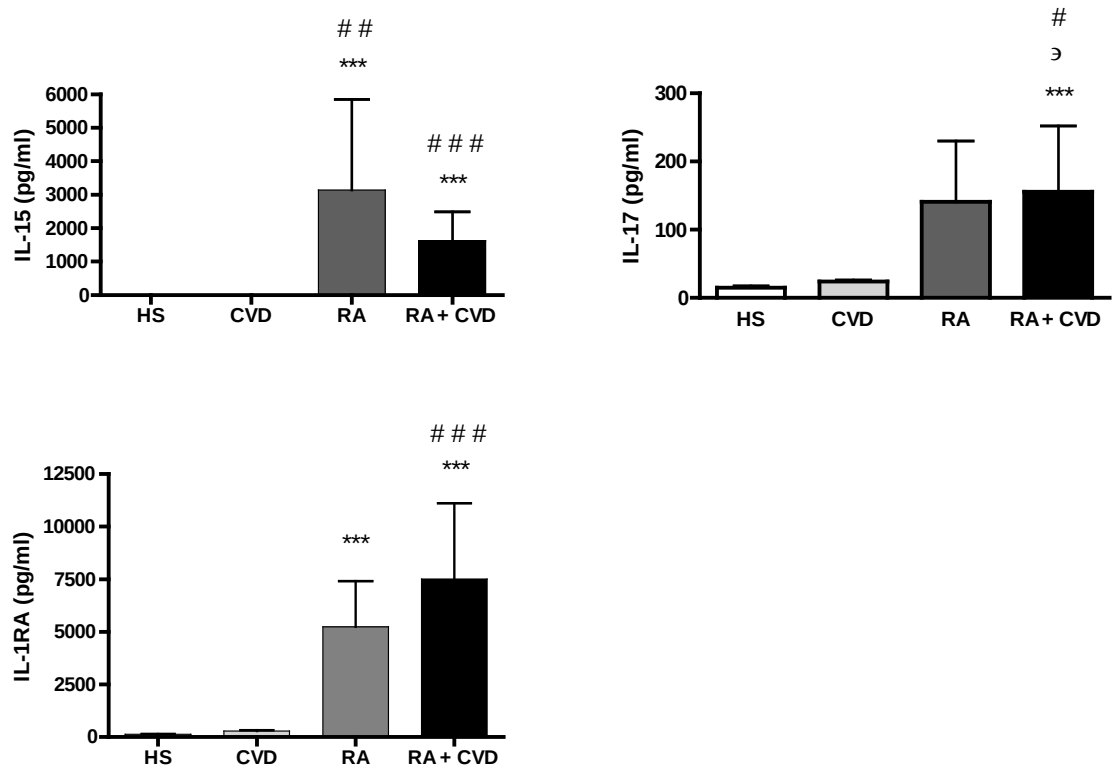


Figure 5.1 Interleukin concentrations in the serum of patients with CVD, RA, RA+CVD and healthy controls

*Serum was isolated from CVD (n=19), RA (n=21), RA+CVD (n=16) and healthy control samples (HS) (n=21). Mean cytokine concentration is expressed as pg/ml and error bars represent ±S.E.M. Statistical analysis was performed by Kruskal-Wallis test followed by Dunn's multiple comparison test. *P<0.05, **P<0.01, ***P<0.001 vs healthy control; #P<0.05, ##P<0.01, ###P<0.001 vs CVD; э P<0.05, ээP<0.01, эээP<0.001 vs RA*

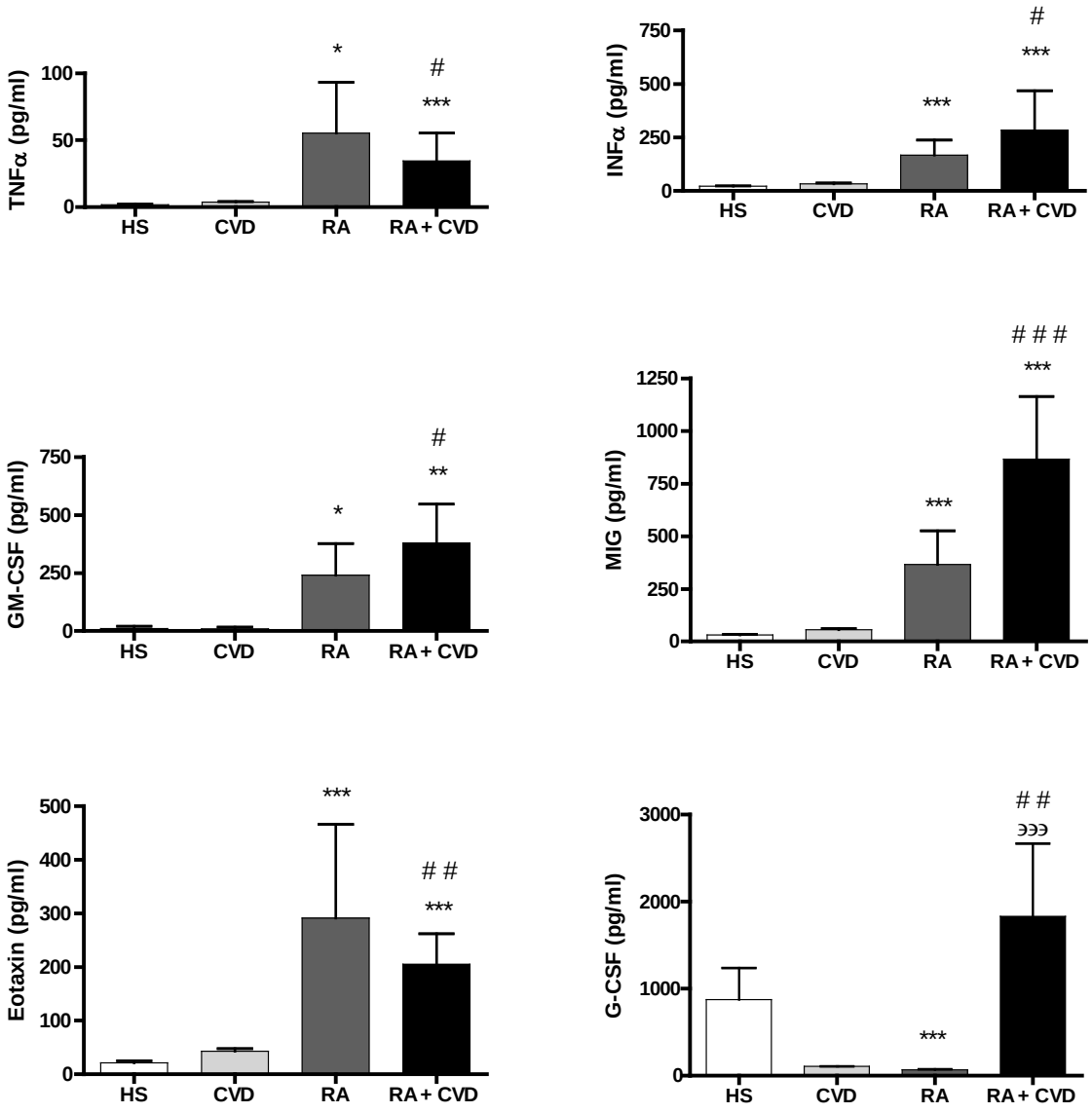


Figure 5.2 Cytokine (non-interleukin) concentrations in the serum of patients with CVD, RA, RA+CVD and healthy controls

*Serum was isolated from CVD (n=19), RA (n=21), RA+CVD (n=16) and healthy control samples (HS) (n=21). Mean cytokine concentration is expressed as pg/ml and error bars represent ±S.E.M. Statistical analysis was performed by Kruskal-Wallis test followed by Dunn's multiple comparison test. *P<0.05, **P<0.01, ***P<0.001 vs healthy control; #P<0.05, ##P<0.01, ###P<0.001 vs CVD; ɜ P<0.05, ɜɜP<0.01, ɜɜɜP<0.001 vs RA*

5.2.2.2 Chemokine and growth factor profile in CVD, RA, RA+CVD subjects

Serum concentrations of six different chemokines (IL-8, MIP-1α, MIP-1β, IP-10, RANTES, MCP-1) and five growth factors (FGF, HGF, IL-7, VEGF, EGF) were analysed in patients with CVD, RA, RA+CVD and healthy subjects.

The RA+CVD cohort had significantly higher serum levels of IL-8 than the RA ($p<0.01$), CVD ($p<0.01$) and healthy cohorts ($p<0.001$) (*figure 5.3*). There was also a significant increase in serum IL-8 levels in RA patients relative to healthy controls. The difference in serum IL-8 levels between the CVD and healthy control cohorts was not statistically significant. These results present the possibility that IL-8 could be used as a biomarker for CVD in RA.

The chemokine RANTES (also known as CCL5) is chemotactic for T cells, eosinophils, and basophiles, and plays an active role in recruiting leukocytes into inflammatory sites. A significant elevation in serum RANTES was observed in RA+CVD patients compared to RA patients. However, a significant increase was also observed in CVD patients compared to RA patients. There was also a significant elevation in serum RANTES in CVD patients relative to healthy controls. Taken together these data suggest that RANTES may be an effective CVD risk biomarker.

Macrophage inflammatory proteins MIP-1α and MIP-1β shared similar expression patterns. These chemokines are produced by activated macrophages resulting in the synthesis and release of various pro-inflammatory cytokines as well as activating granulocytes. Serum levels of the chemokines MIP-1α and MIP-1β were significantly elevated in RA+CVD patients compared to CVD patients and healthy controls, however there was no significant difference observed between the RA+CVD and RA cohorts. MIP-1α and MIP-1β serum levels were also significantly elevated in the RA cohort relative to healthy subjects. There was an apparent increase in the RA patients compared to the CVD patients, although this did not reach statistical significance.

When considering growth factors, it was observed that the serum concentrations of FGF, HGF, IL-7 and EGF were significantly elevated in RA+CVD patients relative to CVD patients and healthy subjects (*figure 5.4*). None of the growth factors analysed were significantly elevated in RA+CVD patients relative to RA patients.

Levels of VEGF (mean ± S.E.M.), an endothelial cell specific growth factor, were 4±4 and 3±3 pg/ml in RA patients and RA+CVD patients respectively, and were below the limits of

detection in CVD patients and healthy subjects. No significant difference was observed between healthy subjects and diseased patients, suggesting that altered systemic concentration of VEGF does not play a role in the pathophysiology of either RA or CVD.

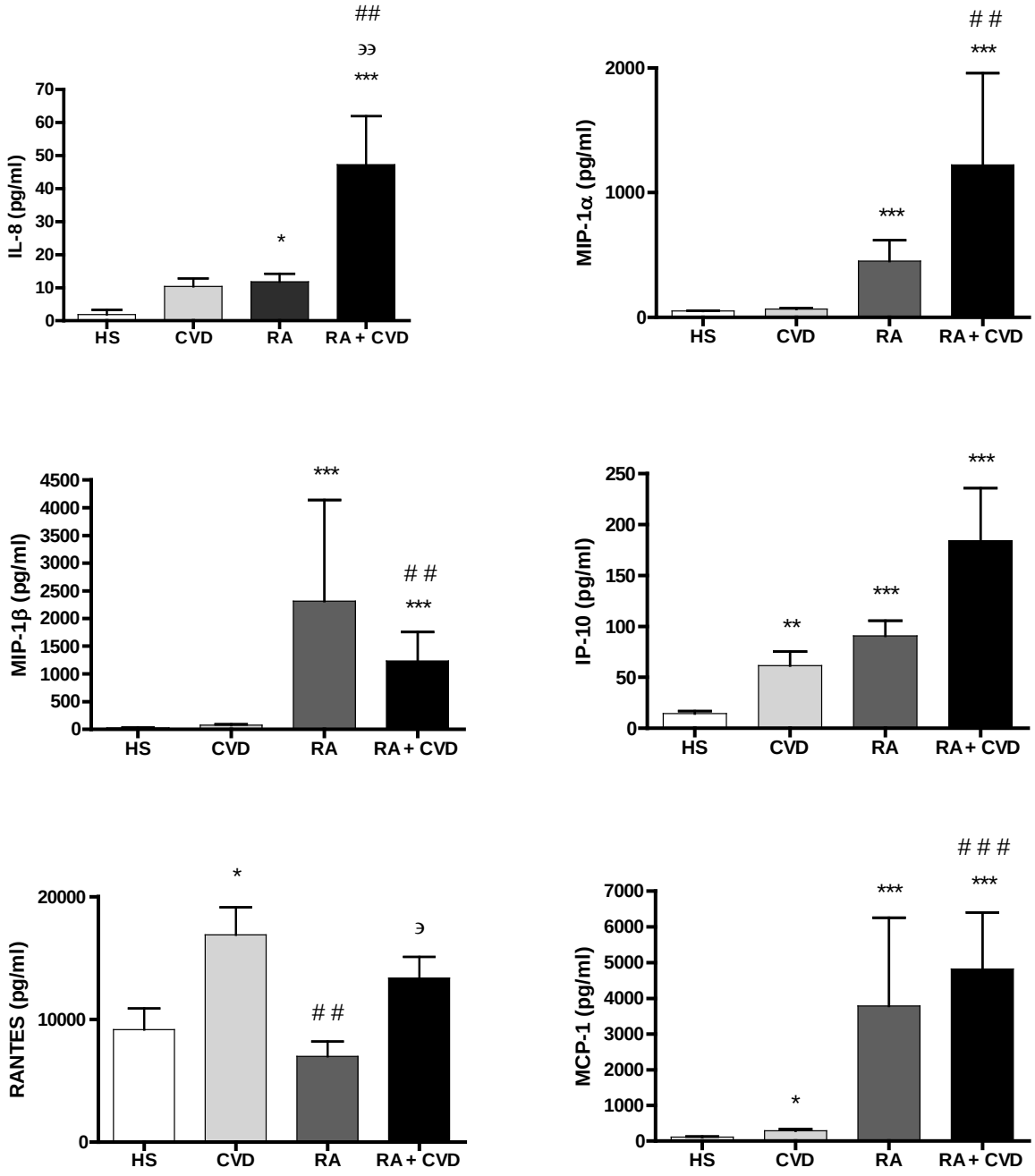


Figure 5.3 Chemokine concentrations in the serum of patients with CVD, RA, RA+CVD and healthy controls

*Serum was isolated from CVD (n=19), RA (n=21), RA+CVD (n=16) and healthy control samples (HS) (n=21). Mean cytokine concentration is expressed as pg/ml and error bars represent ±S.E.M. Statistical analysis was performed by Kruskal-Wallis test followed by Dunn's multiple comparison test. *P<0.05, **P<0.01, ***P<0.001 vs healthy control; #P<0.05, ##P<0.01, ###P<0.001 vs CVD; э P<0.05, ээP<0.01, эээP<0.001 vs RA*

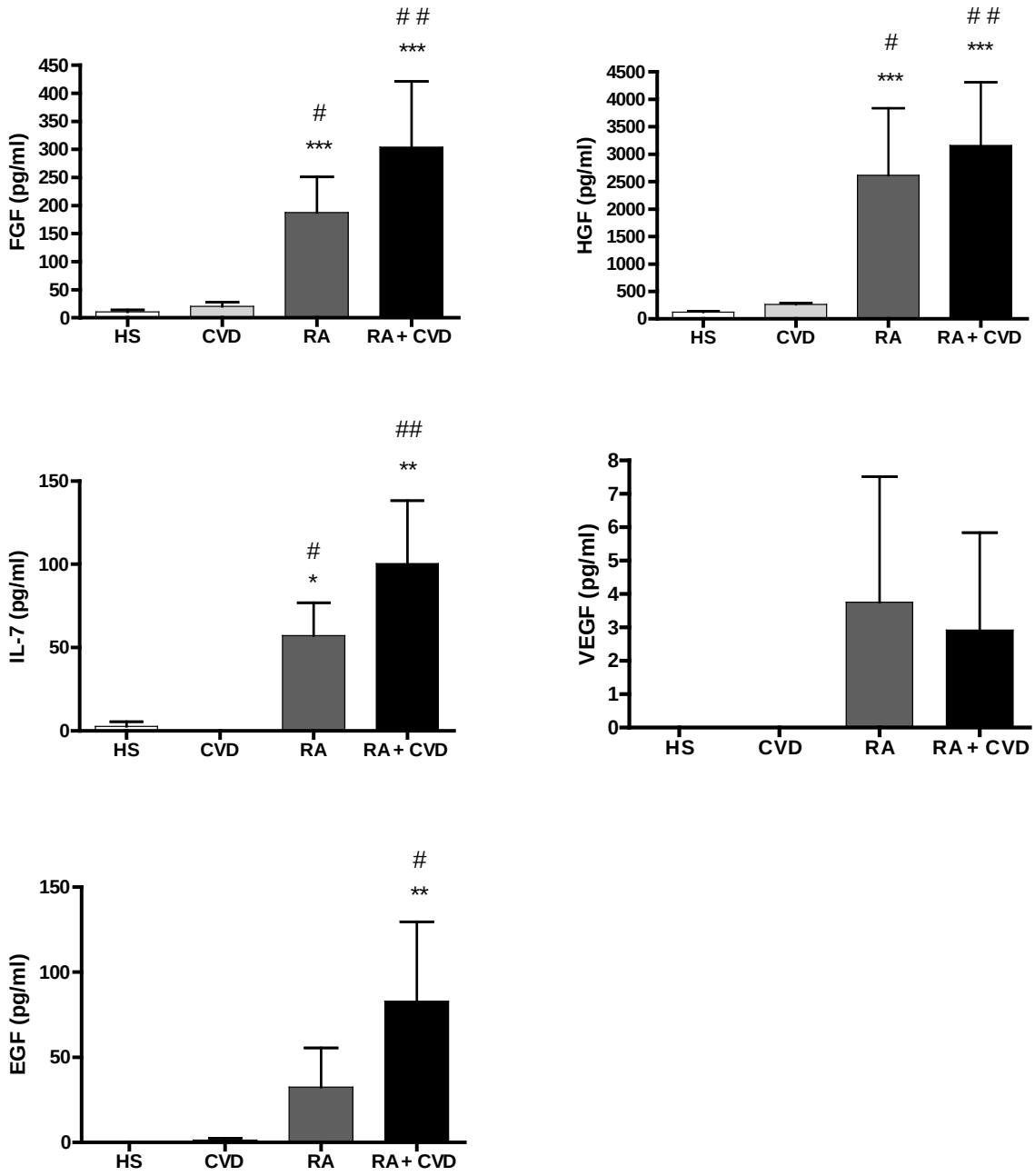


Figure 5.4 Growth factor concentrations in the serum of patients with CVD, RA, RA+CVD and healthy controls

*Serum was isolated from CVD (n=19), RA (n=21), RA+CVD (n=16) and healthy control samples (HS) (n=21). Mean cytokine concentration is expressed as pg/ml and error bars represent ±S.E.M. Statistical analysis was performed by Kruskal-Wallis test followed by Dunn's multiple comparison test. *P<0.05, **P<0.01, ***P<0.001 vs healthy control; #P<0.05, ##P<0.01, ###P<0.001 vs CVD; э P<0.05, ээP<0.01, эээP<0.001 vs RA*

5.2.2.3 sST2 and IL-2R expression in the serum of CVD, RA and RA+CVD subjects

Previously unpublished work from our lab identified IL-33 expression in the aortic adventitia of patients with both CVD and RA (Ahmad, 2010). The receptor for IL-33 is ST2 and the membrane-bound form is constitutively expressed on fibroblasts, mast cells and Th2 cells. A secreted isotype (sST2) is a soluble inhibitory receptor also known as IL1R1A and can be detected in human serum. Binding of IL-33 to the membrane bound ST2 receptor in response to a cardiac event is believed to elicit a cardioprotective function. However, recent studies have shown that sST2 is a decoy receptor that disrupts the interaction of IL-33 with membrane bound ST2. Elevated sST2 levels have been detected in patients early after acute myocardial infarction (Weinberg et al., 2002).

In order to explore the hypothesis that sST2 may play a functional role in the increased CVD burden observed in RA, the serum concentration of sST2 was measured by ELISA in patients with CVD, RA, RA+CVD as well as healthy controls. sST2 was detected in the serum of all four subject cohorts, however there was no significant difference between any of these cohorts ($p>0.05$, Kruskal-Wallis followed by Dunn's multiple comparison) (*figure 5.5*).

Interlekin-2 (IL-2) has a central role in the initiation and development of immune responses through binding to the IL-2 receptor (IL-2R) and serum soluble IL-2R (sIL-2R). The exact biological role of sIL-2R is still not fully understood. It is however known that increased sIL-2R is associated with a broad spectrum of immunological conditions involving B- or T- cell activation and that soluble IL-2R is able to bind IL-2 (Caruso et al., 1993, Boyman and Sprent, 2012). In *section 5.2.2.1* it was determined that IL-2 was significantly elevated in patients with RA relative to CVD patients and healthy controls. In keeping with these previous data, soluble IL-2R was significantly elevated in RA patients relative to healthy controls, and in RA+CVD patients relative to CVD patients (*figure 5.5)*. Unlike IL-2, there was no significant difference in soluble IL-2R expression when considering the RA and CVD cohorts. This can be explained by the large variability in the RA cohort.

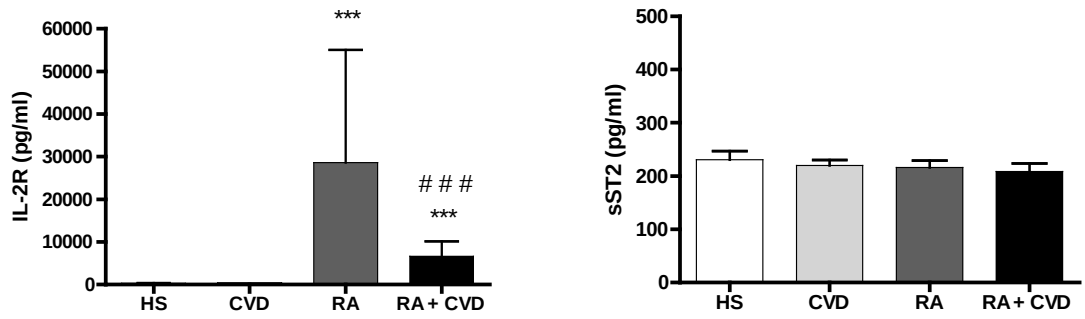


Figure 5.5 sST2 and IL-2R concentrations in the serum of patients with CVD, RA, RA+CVD and healthy controls

*Serum was isolated from CVD (n=19), RA (n=21), RA+CVD (n=16) and healthy control samples (HS) (n=21). Mean concentration is expressed as pg/ml and error bars represent ±S.E.M. Statistical analysis was performed by Kruskal-Wallis test followed by Dunn's multiple comparison test. *P<0.05, **P<0.01, ***P<0.001 vs healthy control; #P<0.05, ##P<0.01, ###P<0.001 vs CVD; э P<0.05, ээP<0.01, эээP<0.001 vs RA. The lower detection limit for each mediator measured was 2pg/ml^{-1}, any values lower than this were considered as 0 for this study.*

5.2.3 Macrophages in the aorta of CVD patients with coexisting RA

Essential to the pathophysiology of atherosclerosis is macrophage accumulation of intracellular lipid, resulting in foam cell formation. Monocytes respond to a broad range of cytokines, chemokines and growth factors that are expressed both systemically and locally by smooth muscle cells, vascular endothelial cells and infiltrated cells. Stimulated monocytes migrate from the peripheral blood into the arterial intima and differentiate into macrophages in the atherosclerotic lesion. Macrophage colony-stimulating factor (M-CSF) is crucial for the differentiation of monocytes into macrophages and for survival of these cells in the atherosclerotic lesion (Takahashi et al., 2002). Macrophages are known to express a variety of surface receptors, predominantly scavenger receptors, which recognise and support endocytosis of modified lipoproteins, including oxidised low-density lipoprotein (oxLDL).

In the previous sections of this chapter (*section 5.2.2 – section 5.2.2.3*) it was established that a variety of cytokines and chemokines are over-expressed in the serum of RA patients. The following data explores the hypothesis that these signals may stimulate macrophages, leading to increased uptake of modified LDL and, consequently, increased foam cell formation.

Firstly, verification that macrophages were present in the aorta of RA+CVD patients was sought. As human atherosclerotic tissue was unavailable, aortic adventia sections from areas with no macroscopic signs of atherosclerosis were stained for CD68, a well established macrophage marker (IHC kindly performed by Ammad Ahmed). $CD68^{+}$ macrophages were detected in 17 of the 19 patients examined and staining was localised to areas of inflammatory cell infiltrate (*figure 5.6*).

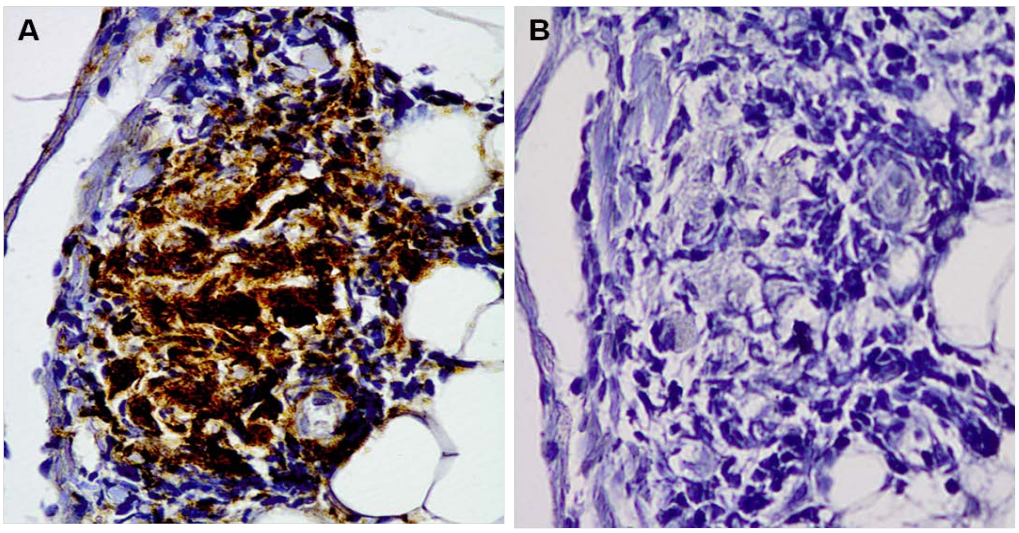


Figure 5.6 Macrophage staining in the aortic adventitia of CVD patients

Aortic adventitial staining for the macrophage marker CD68 can be seen (brown colour) in a lymphocyte aggregate (A). The isotype control showed no background staining (B). Sections were counterstained with haematoxylin and images were captured by light microscopy at 40x magnification.

5.2.4 Low density lipoprotein isolation and oxidative modification

In order to investigate the effect of cytokines and chemokines on oxLDL uptake by macrophages, native LDL was generated from healthy human serum using methods previously developed by Prof. Muriel Caslake (University of Glasgow). This involved the isolation of LDL by sequential flotation ultracentrifugation in a fixed angle rotor.

Following isolation of native LDL it was necessary to quantify the concentration of LDL. This was performed using the modified Lowry protein assay. Briefly, a standard curve was generated using bovine serum albumin (BSA) and the concentration of LDL was then determined by extrapolation from the standard curve. Native LDL was then oxidised by addition of 10µM copper sulphate. Following sufficient reaction lipoproteins were dialysed and stored in PBS containing 0.25mM EDTA. Oxidation was verified by agarose electrophoretic mobility shift assay (*figure 5.7*).

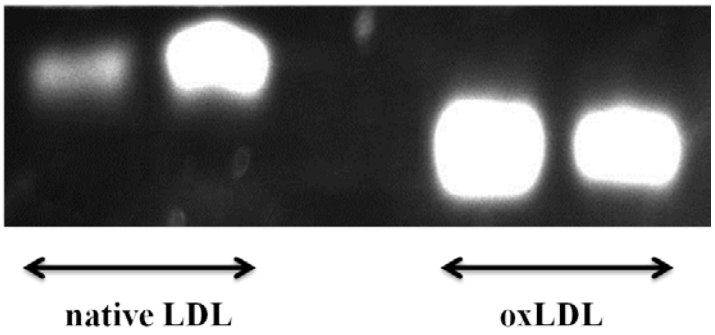


Figure 5.7 Electrophoretic mobility of oxLDL

Native LDL and oxLDL prepared from the same donor were stained with Nile red in duplicate. The LDL preparations were compared by electrophoresis using 1% agarose gel in sodium barbital buffer. Lanes 1 & 2 are native LDL, lanes 3 & 4 are oxLDL.

5.2.5 Kinetics of oxLDL uptake and foam cell formation

Prior to experimentation with oxLDL, it was first necessary to determine the kinetics of foam cell formation in M-CSF derived human primary macrophages. $CD14^+$ monocytes were isolated from human buffy coats and the resulting purity was verified by FACS (>97% purity) as previously outlined (*section 4.10.1*). Monocytes were cultured for 7 days in the presence of 15ng/ml M-CSF to ensure complete differentiation into macrophages. Macrophages were then challenged with different concentrations of oxLDL (2, 20, 80, 200 and 300µg/ml) for 24 hours (n=3). Internalised oxLDL was visualised using Oil-red-O stain and the number of foam cells counted. The percentage of macrophages exhibiting foam cell morphology increased with increasing oxLDL concentration (*figure 5.8*). Maximal foam cell formation was observed following incubation with 200µg/ml oxLDL. Having established that 200µg/ml oxLDL supported maximal foam cell formation, it was then necessary to quantify the kinetics of foam cell formation. Macrophages were challenged with 200µg/ml oxLDL for 1, 2, 3, 4, 8, 12 and 24 hours. Following one hour incubation, approximately 75% of macrophages exhibited foam cell morphology with saturation being reached after three hours (*figure 5.8*). Although approximately 100% of macrophages were converting to foam cells following three hours incubation with 200µg/ml oxLDL, the intensity of Oil-red-O staining increased for the 24-hour duration of the experiment (*figure 5.8*), indicating that lipid accumulation continued.

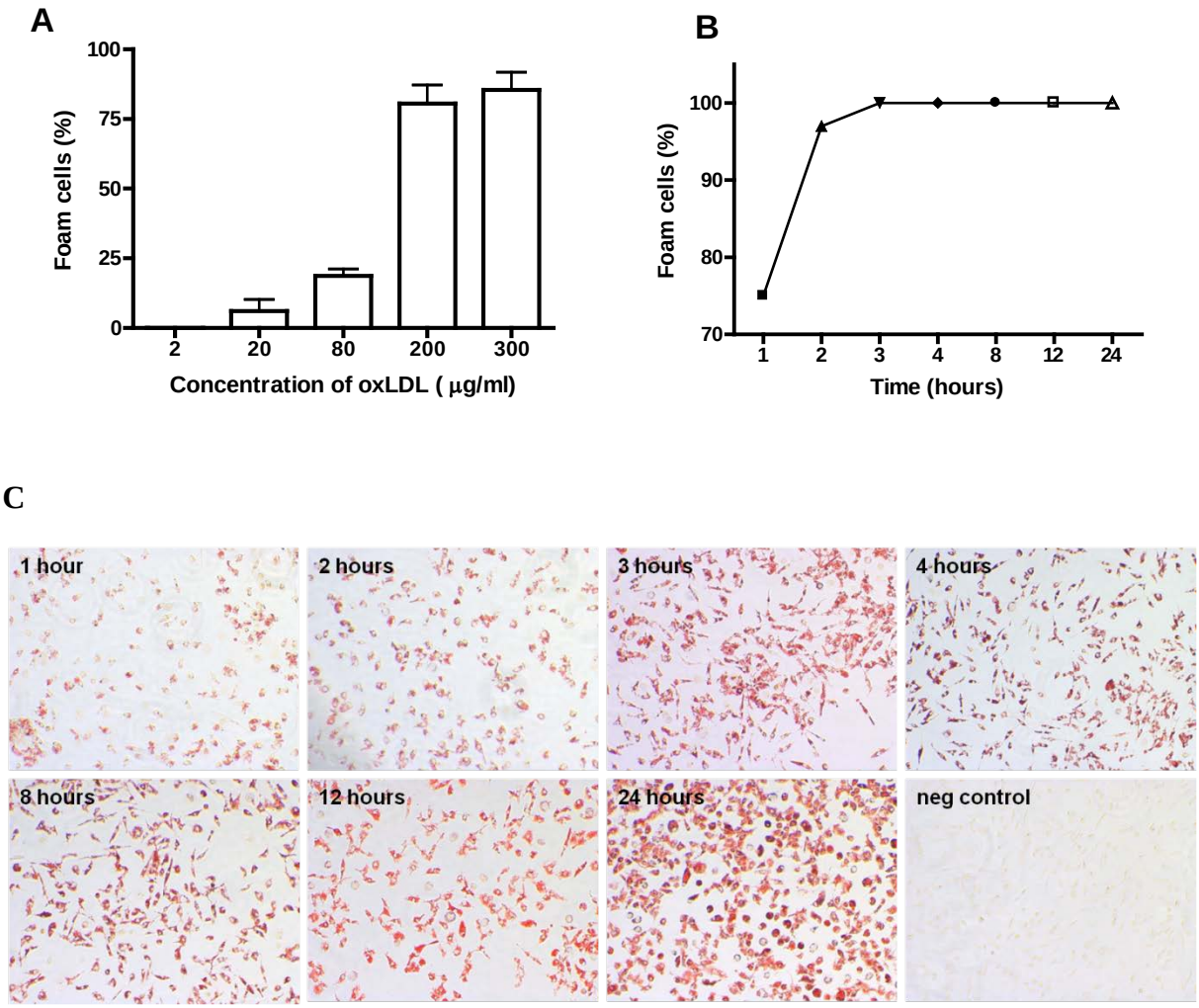


Figure 5.8 Kinetics of oxLDL uptake by human macrophages

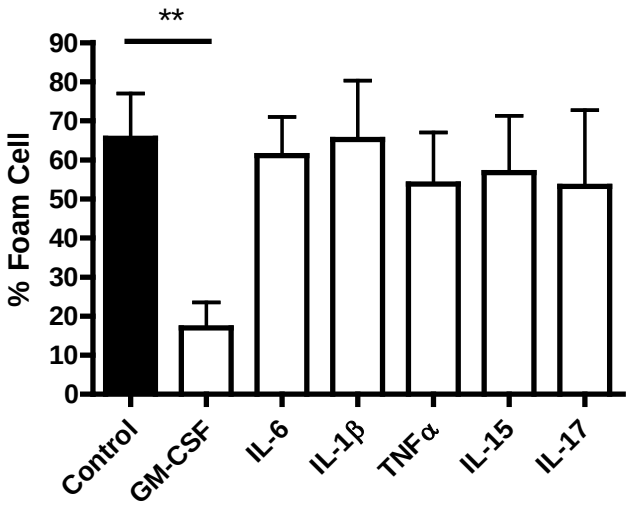
(A) Concentration-dependent curve of foam cell formation induced by oxLDL (n=3). Human primary macrophages were incubated with different concentrations (2, 20, 80, 200 and 300µg/ml) of oxLDL for 24 hours. Following Oil-Red-O staining, the percentage of macrophages exhibiting foam cell like morphology was calculated. (B) Time-dependent curve of foam cell formation induced by oxLDL (n=2). Human primary macrophages were incubated with 200µ/ml oxLDL for different lengths of time (1, 2, 3, 4, 8, 12 and 24 hours) and the percentage of macrophages exhibiting foam cell morphology determined. (C) Representative images of the formation of foam cells over time (original magnification 100x).

5.2.6 OxLDL loading in cytokine-treated human monocyte-derived macrophages

Primary human macrophages were maintained in the presence of 15ng/ml M-CSF for seven days. Cells were then stimulated overnight with GM-CSF (125ng/ml), IL-6 (50ng/ml), IL-1β (5ng/ml), TNFα (50ng/ml), IL-15 (50ng/ml) or IL-17 (125ng/ml), all of which have been implicated in RA and CVD pathogenesis. Control cells were stimulated with M-CSF (15ng/ml) only. We previously demonstrated that these RA-associated cytokines were all significantly elevated in RA patients relative to healthy controls (*section 5.2.2.1*). Following overnight stimulation, cells were challenged with 200µg/ml oxLDL for 1 hour. Subsequent Oil-red-O staining allowed the percentage of macrophages exhibiting foam cell morphology to be accurately determined.

In keeping with previous kinetics experiments (*section 5.2.5*) approximately 70% of un-stimulated control macrophages formed foam cells. In contrast, only ~15% of macrophages stimulated with the growth factor GM-CSF formed foam cells. GM-CSF significantly reduced the foam cell formation to 25% of control cells. IL-6, IL-1β, TNFα, IL-15 and IL-17 had no significant effect on foam cell formation (*figure 5.9*).

A



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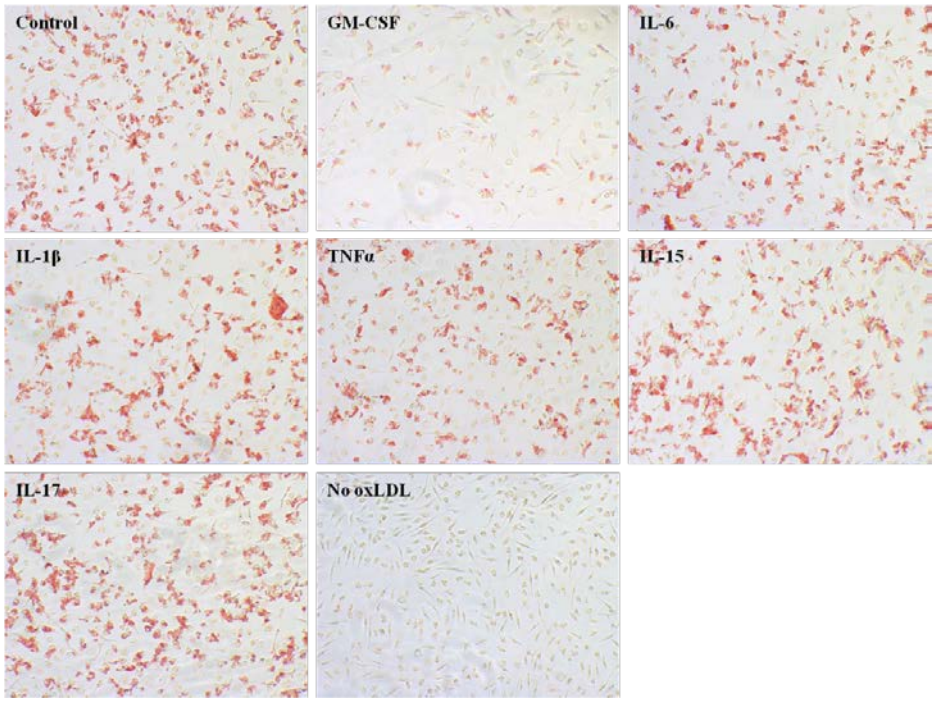

Figure 5.9 OxLDL loading in cytokine-treated human monocyte-derived macrophages *(A) Human monocyte derived macrophages were maintained in M-CSF and then stimulated overnight with GM-CSF (125ng/ml), IL-6 (50ng/ml), IL-1β (5ng/ml), TNFα (50ng/ml), IL-15 (50ng/ml) or IL-17 (125ng/ml). Control cells were maintained in M-CSF only. Macrophages were then challenged with 200µg/ml oxLDL for one hour and the percentage of cells expressing foam cell morphology determined following Oil-red-O staining. Data are representative of three experiments ± SD. Statistical analysis was performed by Kruskal-Wallis test followed by Dunn's multiple comparison test.* $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. *(B) Visual representation of Oil-red-O staining.*

5.2.7 The effects of GM-CSF on human macrophage responses

In the previous section (*section 5.2.6*) it was demonstrated that GM-CSF inhibited both uptake of oxLDL by macrophages and the subsequent formation of foam cells. However, as GM-CSF is a pleiotropic growth factor it likely affects several macrophage differentiation and effector functions which may contribute to the biological effects observed.

To determine if GM-CSF caused downstream alteration in the immune profile, human monocyte-derived macrophages were stimulated overnight with GM-CSF or vehicle control and protein levels in the supernatant were measured by Luminex assay.

GM-CSF was found to significantly increase the expression of 17 of the 23 proteins analysed (*figure 5.10*). The majority of these were pro-inflammatory (IL-2, IL-4, IL-5, IL-6 and MIP-1α), however the expression of the anti-inflammatory molecules, IL-10 and IL-1RA were also found to be significantly increased. Expression of the growth factor VEGF was also found to significantly increase following GM-CSF stimulation. Although the implications for VEGF in foam cell formation remain unknown, this protein has been established as a potent angiogenic factor (Celletti et al., 2001) and may have implications for CVD acceleration in RA patients who over-express GM-CSF. Interestingly GM-CSF was also found to significantly increase the expression of IL-15 and IL-17, although it was shown that they do not alter the rate of foam cell formation (*section 5.2.6*)

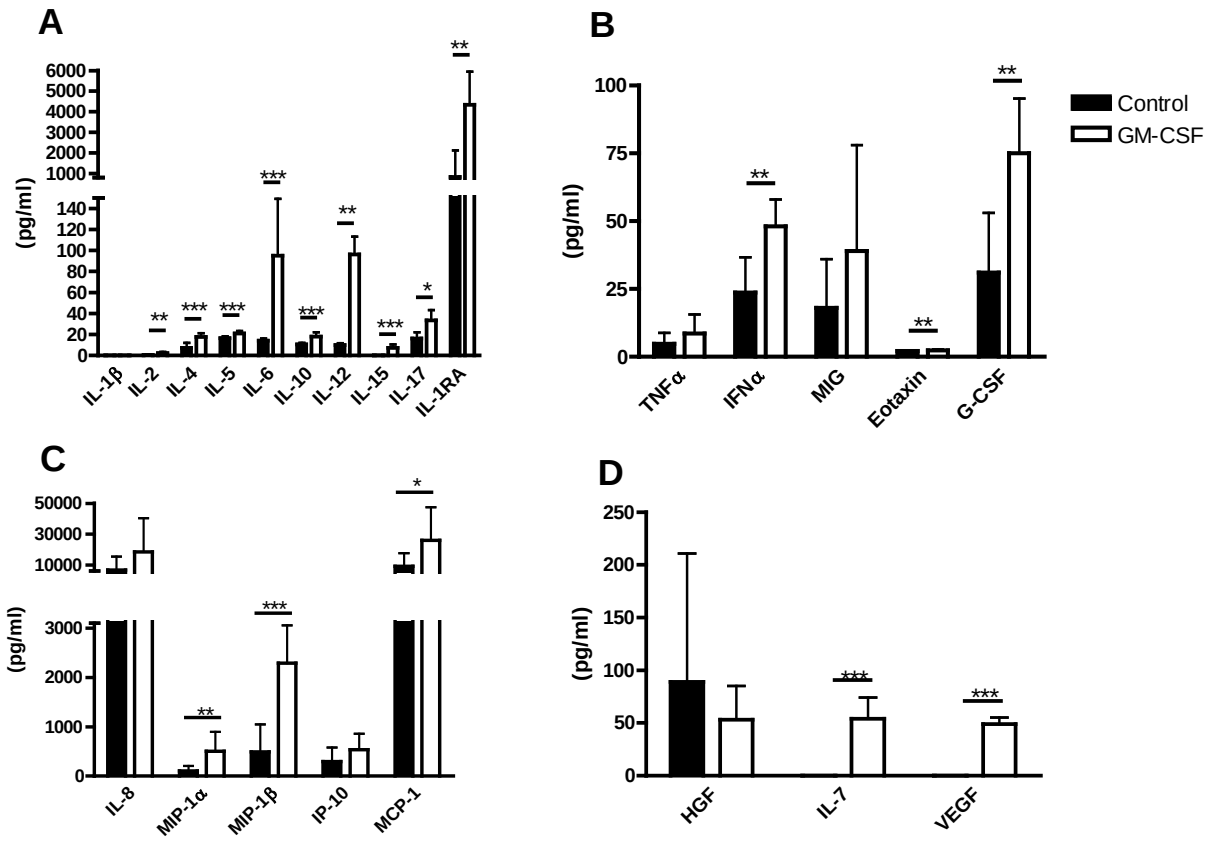

Figure 5.10 Immune profile of GM-CSF-stimulated human macrophages

Human monocyte-derived macrophages were maintained in 15ng/ml M-CSF and then stimulated overnight with GM-CSF (125ng/ml). Control cells were maintained in M-CSF and stimulated with vehicle control only. Cell culture supernatants were removed and protein concentration measured by LUMINEX assay. GM-CSF stimulated (n=6); control (n=11). (A) cytokines (interleukins); (B) cytokines (non-interleukin); (C) chemokines and (D) growth factors. Error bars illustrate standard deviation. Statistical analysis was performed by unpaired t-test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

5.2.8 Inhibition of foam cell formation using dextran sulphate

Macrophages are thought to endocytose modified lipoproteins, including oxLDL, via scavenger receptors, resulting in accumulation of cholesterol ester and subsequent foam cell formation (Dhaliwal and Steinbrecher, 1999). It is necessary to verify that scavenger receptors are responsible for the uptake of oxLDL observed in the previous experiments, and to determine if GM-CSF alters the kinetics of scavenger receptor-mediated oxLDL endocytosis. To test these underlying hypotheses, samples were treated with varying amounts of dextran sulphate, a compound known to competitively bind and inhibit the function of scavenger receptors (Tsubamoto et al., 1994).

Maximal foam cell formation was first ensured by incubating GM-CSF stimulated and control macrophages with 200µg/ml oxLDL for 24 hours. *Figure 5.11* demonstrates that this time point supported maximal foam cell formation. It is worth noting, despite ~100% of GM-CSF- stimulated and control cells transforming into foam cells over this time period, that the GM-CSF-stimulated macrophages appeared to have taken up less oxLDL overall. Although this was not quantified, it is visible in *figure 5.12* as a reduction in Oil-red-O staining intensity.

GM-CSF and control cells were then incubated with 200µg/ml oxLDL and varying concentrations of dextran sulphate (2, 10, 20, 40µg/ml) for 24 hours. As the concentration of dextran sulphate was increased from 0 to 40µg/ml the inhibition of foam cell formation increased (*figure 5.11*), indicating that scavenger receptor-mediated endocytosis plays a vital role in oxLDL uptake and foam cell formation. Interestingly, dextran sulphate appeared to significantly decrease the extent of foam cell formation in GM-CSF-stimulated macrophages relative to control macrophages. For example treatment with 10µg/ml dextran sulphate resulted in 5±8.6% and 44±13% inhibition of foam cell formation in control cells and GM-CSF stimulated cells, respectively.

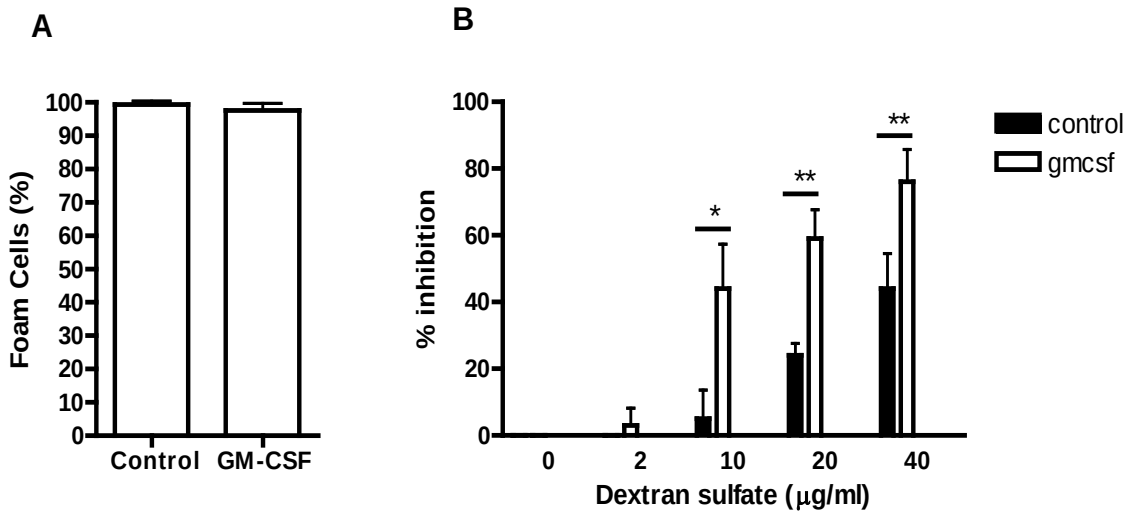

Figure 5.11 Competitive inhibition of scavenger receptor-mediated uptake of oxLDL

(A) Human monocyte-derived macrophages were maintained in 15ng/ml M-CSF and then stimulated overnight with GM-CSF (125ng/ml). Control cells were maintained in M-CSF and stimulated with vehicle control only. Macrophages were then challenged with 200μg/ml oxLDL for 24 hours. The percentage of cells expressing foam cell morphology was determined following Oil-red-O staining. Cells in (B) were treated in a similar manner with the addition of varying concentrations of dextran sulphate (0, 2 10, 20 and 40μg/ml). The percentage of cells expressing foam cell morphology was determined following Oil-red-O staining. Graphed data represent percentage foam cell inhibition relative to macrophages incubated with 200μg/ml oxLDL for 24 hours but no dextran sulphate. Data are representative of three experiments ± SD. Statistical analysis was performed by unpaired t-test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

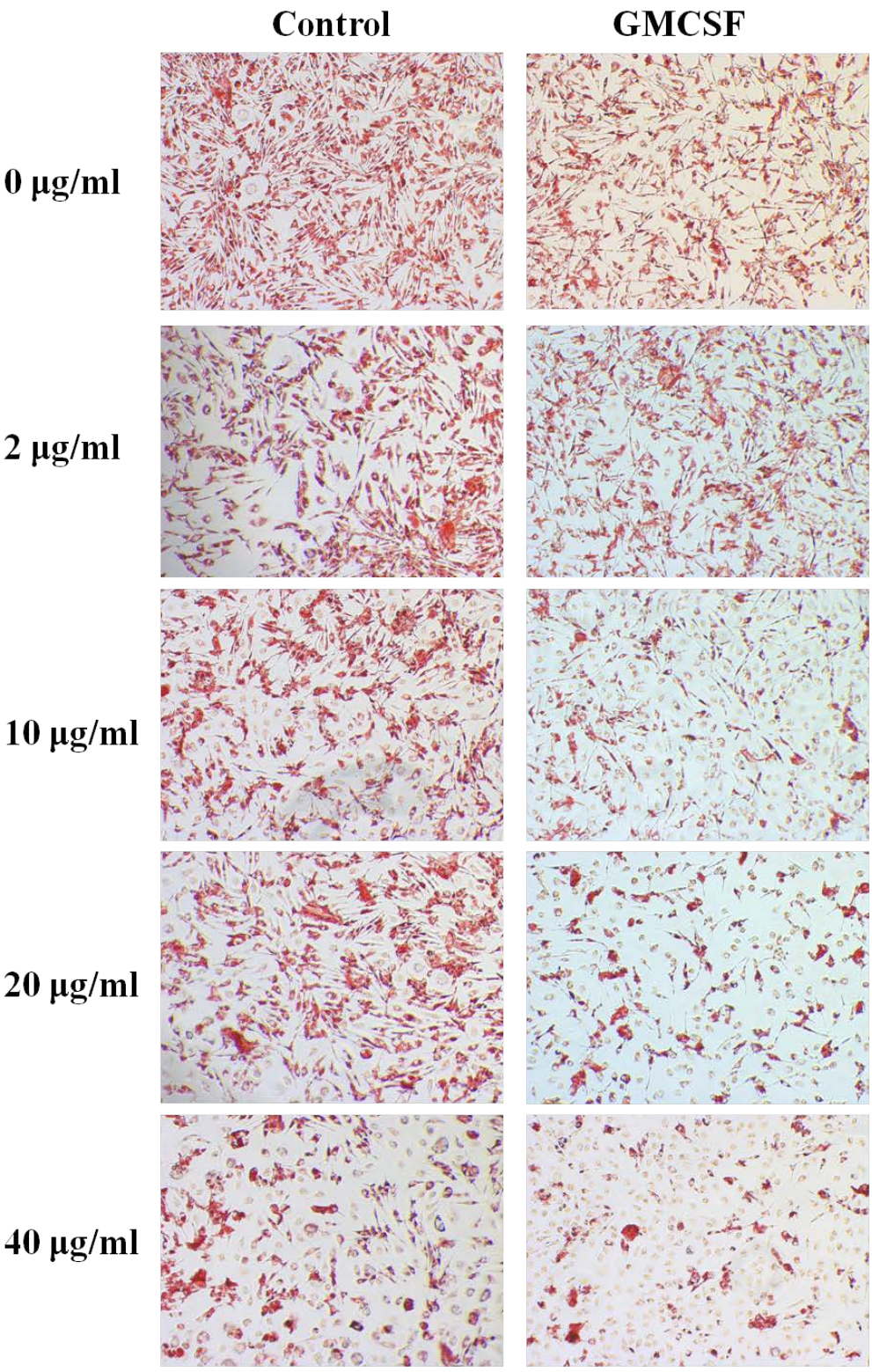

Figure 5.12 Oil-red-O staining following inhibition of scavenger receptor mediated uptake of oxLDL

Representative images of data used to generate figure 5.11. Images were captured by light microscopy at 100x magnification.

5.2.9 Scavenger receptor expression in cytokine-treated human monocyte-derived macrophages

In *section 5.2.8* it was determined that GM-CSF-activated macrophages required a lower concentration of dextran sulphate (a competitive inhibitor of scavenger receptors) to inhibit oxLDL uptake and subsequent foam cell formation. This finding suggests that GM-CSF activated macrophages either express less total scavenger receptors on the surface or express less of a specific scavenger receptor that possesses high affinity for oxLDL. Alternatively, although less likely, GM-CSF-stimulated macrophages may express scavenger receptors that are hypersensitive to inhibition by dextran sulphate.

In order to investigate this further the transcriptional activity of SR-A1, SR-B1, CD36 and MARCO was analysed (*figure 5.13*). Each of these receptors are known to recognise oxLDL and are thought to be the principal receptors responsible for the uptake of modified LDL. Flow cytometry was also used to investigate the expression of SR-B1 and MARCO at a protein level. For completeness, macrophages stimulated with GM-CSF as well as TNFα, IL-6, IL-1β, IL-15 and IL-17 were studied.

GM-CSF-activated macrophages exhibited significantly greater transcriptional activity for SR-B1 relative to control cells (11±7 fold change) (*figure 5.13, B*), however this was not observed at the protein level (*figure 5.14, B*). The transcription of SR-A1 and CD36 remained unchanged by overnight stimulation with GM-CSF. GM-CSF activated macrophages exhibited a trend towards transcriptional down regulation (-5.6-fold) of MARCO, however this did not reach significance. Furthermore, this trend was not observed at a protein level.

Interestingly, it was observed that TNFα and IL-6 significantly decreased the transcription of genes encoding SR-A1, SR-B1 and CD36, however this was not observed at the protein level (*figure 5.14*).

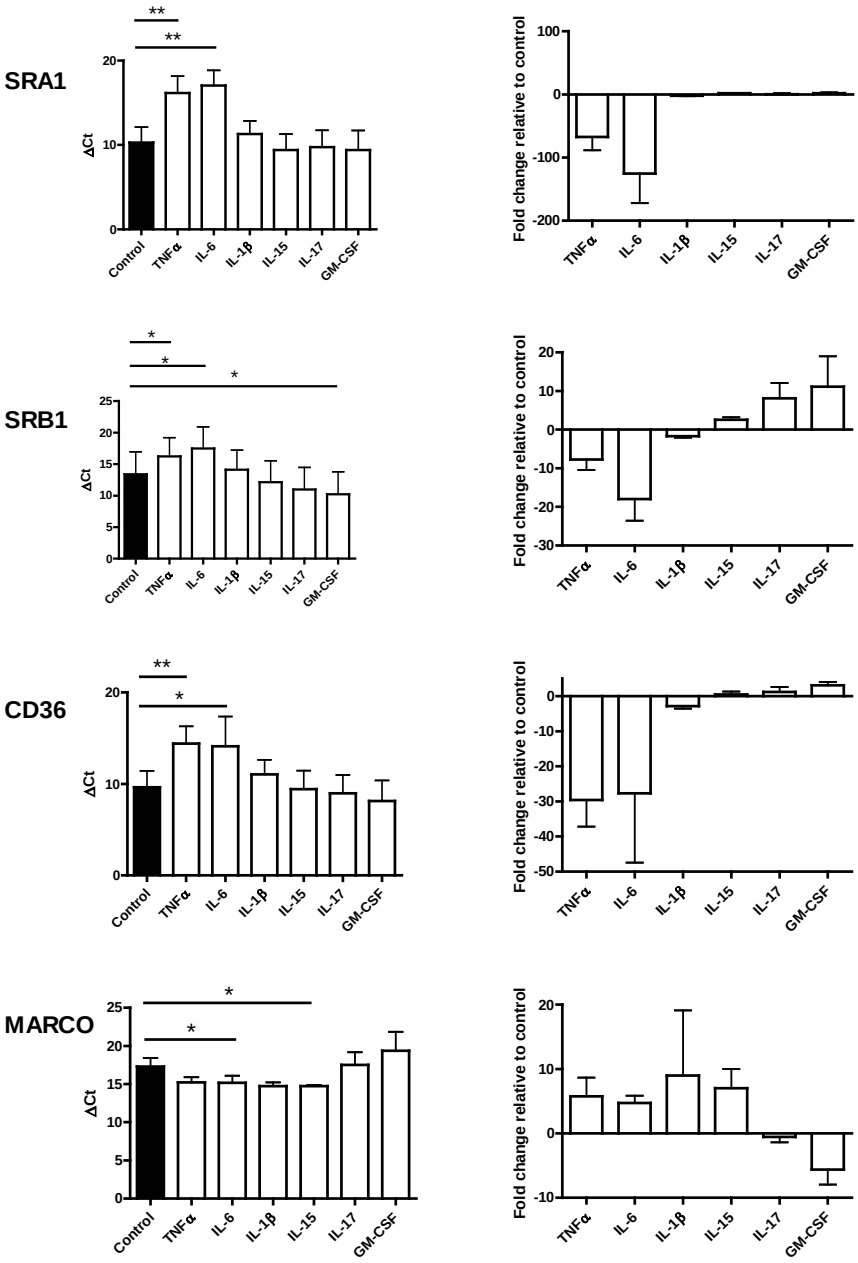

Figure 5.13 mRNA expression of scavenger receptors in cytokine treated human monocyte-derived macrophages

Human monocyte-derived macrophages were maintained in M-CSF and then stimulated overnight with TNFα (50ng/ml), IL-6 (50ng/ml), IL-1β (5ng/ml), IL-15 (50ng/ml), IL-17 (125ng/ml) or GM-CSF (125ng/ml). Control cells were maintained in M-CSF only (black bar). The relative abundance of SRA1, SRB1, CD36 and MARCO was determined by qPCR. Left hand figures represent Ct values following normalisation to the housekeeping gene 18S. Right hand figures represent fold change relative to control cells. Data are representative of three experiments ± SD. Statistical analysis was performed on Ct values by paired t-test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

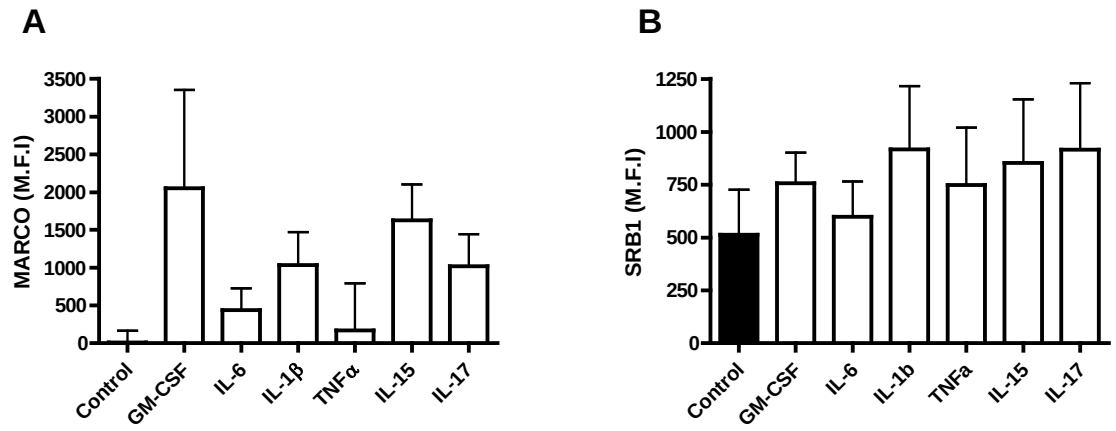

Figure 5.14 FACS staining of MARCO and SRB1 in cytokine-treated human monocyte derived macrophages

Human monocyte derived macrophages were maintained in M-CSF and then stimulated overnight with TNFα (50ng/ml), IL-6 (50ng/ml), IL-1β (5ng/ml), IL-15 (50ng/ml), IL-17 (125ng/ml) or GM-CSF (125ng/ml). Control cells were maintained in M-CSF only (black bar). Cells were harvested and stained with primary antibodies which recognise extracellular MARCO (graph A) and total SRB1 (graph B). Bar graphs show expression of scavenger receptors normalised to isotype-matched control antibodies. M.F.I: Mean Fluorescent Intensity. The results shown are representative of three independent experiments ± SD. Statistical analysis was performed for each condition relative to control by paired t-test.

5.3 Discussion

In RA, the primary site of inflammation is the synovial tissue, from which cytokines are released into systemic circulation. These circulating cytokines have been suggested to have an effect upon distant tissues, including liver, skeletal muscle, adipose, and vascular endothelium, to generate a variety of proatherogenic changes that include dyslipidaemia, insulin resistance, pro-oxidative effects, and endothelial dysfunction (Sattar et al., 2003). As such, systemic inflammation has been hypothesised to represent the missing link that could account for the higher burden of CVD observed in RA patients (Szekanecz and Koch, 2008). Previous studies have mostly focused on the general inflammatory markers (e.g. CRP, ESR and leukocyte counts) as well as traditional inflammatory cytokines (e.g. TNFα and IL-6), and some studies have been published which characterise differences in CVD and RA+CVD patients (Breland et al., 2010). The present study built upon previous findings by analysing the serum of patients with RA, CVD, RA+CVD and healthy control subjects for a range of 30 cytokines, chemokines, growth factors and receptors.

The principle finding of this study was that serum levels of several immuno-proteins are elevated in RA+CVD patients relative to RA patients and CVD patients. By examining a wide range of proteins it was shown that the mean concentration of eight cytokines (IL-1β, IL-2, IL-5, IL-13, IL-17, MIG, G-CSF and GM-CSF) and the chemokine IL-8 was greater in the RA+CVD cohort compared to the additive values of the RA only and CVD only cohorts. However, only IL-17, IL-8 and G-CSF were found to be significantly greater in the RA+CVD groups relative to both the CVD and RA groups. These data lead to the hypothesis that these bio-markers, either individually or cumulatively, may form a pro-inflammatory phenotype in RA which increases the risk of CVD.

Many of these biomarkers have already been studied in the context of CVD; however, their exact role(s) in pathogenesis often remains elusive. Studies have shown that knock-out of IL-1β and treatment with anti-IL-2 decreases the severity of atherosclerosis in Apo-E deficient mice (Kirii et al., 2003, Upadhya et al., 2004). GM-CSF is also thought to be pro-atherosclerotic, with experiments showing that treatment of mice with GM-CSF increased cell proliferation in early lesions, whereas treatment with anti-GM-CSF inhibited proliferation (Zhu et al., 2009). Conversely IL-5, as a result of its ability to expand natural IgM specific for oxLDL, has been demonstrated as having atheroprotective properties (Binder et al., 2004). Recent studies have shown that IL-17 plays a pro-atherosclerotic role

(van Es et al., 2009, Erbel et al., 2009), where as a previous study suggests IL-17 is atheroprotective (Taleb et al., 2009). Thus, the function of IL-17 remains controversial and requires additional direct studies. Currently, little is known regarding the role that IL-13 and MIG play in the pathogenesis of atherosclerosis

G-CSF exhibited an unusual and unique expression pattern, with CVD and RA patients having considerably lower serum concentrations (105 and 66 pg/ml, respectively) than healthy and RA+CVD subjects (875 pg/ml and 1832 pg/ml, respectively). If assuming that healthy patients have normal serum levels of G-CSF, it would appear that G-CSF is reduced in both CVD and RA, but remains relatively unchanged in RA+CVD patients. Although not investigated here, it would be of value to determine whether RA patients return to a 'normal' G-CSF concentration upon developing CVD, or whether these patients have always possessed a different G-CSF profile, which could increase CVD risk. Unfortunately, examination of the literature does not raise any reasonable hypotheses, and does not lend itself to speculation.

The chronic inflammatory state observed in CVD is believed to result, at least in part, from immune cells such as monocytes being continually recruited to the atherosclerotic lesion. Macrophages are capable of ingesting modified LDL to form cholesterol-rich foam cells, which become the initial building blocks of atherosclerotic plaques. However, an understanding of how the chronic inflammatory state observed in RA modulates macrophage foam cell formation is lacking.

In *section 5.2.6* it was demonstrated that GM-CSF stimulation significantly decreased the rate at which human primary macrophages could endocytose oxLDL. It was further shown that TNFα, IL-6, IL-1β, IL-15 and IL-17 had no significant effects on the rate of oxLDL uptake. Although clearly a very interesting result, these data have several limitations. Firstly, the GM-CSF effect was observed in isolation. It is possible that in an environment containing an array of cytokines and chemokines, more closely representing human serum, the GM-CSF effect would be reduced or possibly eliminated. Furthermore, in *section 5.2.7* it was demonstrated that GM-CSF stimulation significantly increases macrophage expression of several other cytokines and chemokines. Of particular note, both MIP-1α and MIP-1β were significantly elevated following GM-CSF stimulation. These chemokines are known for their chemotactic and pro-inflammatory effects but can also promote homeostasis (Maurer and von Stebut, 2004). The cytokine G-CSF, which shares many properties with GM-CSF, was also significantly increased. G-CSF has been

previously demonstrated to have a protective effect in a rabbit atherosclerosis model. In this model G-CSF prevented an increase in neointima/media ratio and accelerated reendothelialisation (Hasegawa et al., 2006). It is thus possible that GM-CSF stimulation *per se* is not responsible for the reduction in oxLDL uptake, but instead GM-CSF stimulates production of other proteins which may illicit the observed effect. Alternatively, this biological event may be a result of the additive function of the protein milieu secreted in response to GM-CSF stimulation. To fully understand the effect of elevated serum GM-CSF on atherosclerosis and foam cell formation, biologically relevant animal modelling would be necessary.

In *section 5.2.8* the effect of dextran sulphate, a known competitive inhibitor of scavenger receptors, on the ability of M-CSF and GM-CSF stimulated macrophages to produce foam cells was analysed. This study demonstrated that GM-CSF decreases the concentration of dextran sulphate required to successfully inhibit scavenger receptor-mediated ox-LDL uptake and subsequent foam cell formation. This would suggest that GM-CSF-stimulated macrophages either express less scavenger receptors on the cell surface or express scavenger receptors that are hyper-sensitive to inhibition by GM-CSF. Initial investigations into the expression of SR-A1, SR-B1, CD36 and MARCO showed that none of these scavenger receptors were decreased at a transcriptional level following stimulation with GM-CSF.

GM-CSF was originally characterised as a haematopoietic growth factor responsible for the differentiation of bone marrow progenitor cells (Burgess and Metcalf, 1980), however more recently it has also been established as a cytokine with important roles in both inflammation and infection (Shi et al., 2006). Furthermore, a variety of studies have shown that GM-CSF promotes survival and activation of macrophages (Hamilton, 2008, Hamilton et al., 1980). Although, the present study determined that GM-CSF protected from foam cell formation, it did not definitively show how such protection could occur. As a broad generalisation, in *in vitro* studies, GM-CSF preferentially generates, either alone or in the presence of M-CSF, populations with dendritic cell like and antigen presenting capabilities, whereas the progeny arising from M-CSF stimulation are phagocytic macrophage like cells (Fleetwood et al., 2007). Surprisingly, despite this apparent shift to from ‘phagocytic’ to ‘antigen presenting’, no decrease in scavenger receptor transcript was detected following GM-CSF stimulation. In order to further hypothesise as to why GM-CSF reduces foam cell formation, it would be useful to further consider the differences between macrophages stimulated with M-CSF and GM-CSF.

Several studies suggest that GM-CSF has a clear pro-inflammatory effect on macrophage populations and that GM-CSF expression is elevated at sites of inflammation (Sorgi et al., 2012). By contrast, macrophages are continually exposed to M-CSF as it is expressed constitutively during times of homeostasis. Such macrophages have a comparably compromised ability to secrete pro-inflammatory mediators and other toxic intermediates, such as nitric oxide, but possess a capacity to promote tissue repair and angiogenesis (Fleetwood et al., 2007). It is possible that M-CSF-stimulated macrophages in the 'steady-state' have a phenotype which promotes effective scavenging of their environment and subsequent phagocytosis. Alternatively, following a GM-CSF mediated shift to a pro-inflammatory state, macrophages may favour activities such as recruitment of other immune cells and antigen presentation and expend less energy on processes such as phagocytosis.

It has also been noted that there are differences in nuclear factor-κB (NF-κB) activity and RAS signalling pathways between M-CSF- and GM-CSF-stimulated macrophages (Fleetwood et al., 2007). It is possible that these pathways determine alternative surface receptor expression and/or phagocytic activity. Further experimentation is required to test this hypothesis.

The current study set out to explore mechanisms by which RA increases the risk of CVD, but instead has identified GM-CSF, which is elevated in RA serum, as having potential atheroprotective properties. Recently, however, antagonism of GM-CSF has been suggested as a novel therapeutic approach for a variety of autoimmune diseases, including RA (Cornish et al., 2009). Phase I human clinical trials with Mavrilimumab, a human monocolonal antibody targeting GM-CSF receptor-α, demonstrated pharmacodynamic activity (Burmester et al., 2011). Although it is still too early to say whether this therapeutic will make it to the market, these preliminary studies demonstrates both safety and an adequate pharmacokinetic profile and supports further clinical study in RA. Novel RA therapeutics are increasingly necessary to treat the significant proportion of patients who fail to achieve minimum improvement criteria. However the current study raises the possibility that anti-GM-CSF therapy would support increased foam cell formation and may have a potential long term detrimental cardiovascular effect. Further studies would be needed to demonstrate conclusively whether anti-GM-CSF therapy has an adverse cardiovascular effect.

As already mentioned, the present study found TNFα to have no effect upon foam cell formation. This was not entirely surprising as, despite a significant transcriptional down-regulation of the scavenger receptors SR-A1, SR-B1 and CD36 being detected, no decrease in either SR-B1 or MARCO was observed at a protein level. Previous studies have however shown TNFα to suppress macrophage scavenger receptor (specifically SR-A1) function and reduce subsequent foam cell formation (Vanlenten and Fogelman, 1992). It has been suggested that TNFα regulates scavenger receptor expression by transcriptional and post-transcriptional mechanisms, but principally by destabilisation of scavenger receptor mRNA (Hsu et al., 1996). The inconsistency between these data could have several explanations. Importantly the present study was performed with primary human macrophages isolated from several different donors and differentiated in M-CSF, whereas Vanlenten & Fogelman (1992) used the human monocytic macrophage THP-1 cell line and differentiated in the presence of phorbol 12-myristate 13-acetate (PMA). The functional consequences of using a PMA differentiated macrophage cell line instead of M-CSF derived human primary macrophages are relatively unknown, but it is likely that differences in scavenger receptor expression and phagocytosis activity exist. Furthermore, the present study challenged macrophages with oxLDL whereas previous studies treated with acetylated LDL. Evidence indicates that oxLDL, rather than acLDL, is the physiological representative of modified LDL (Palinski et al., 1989). Furthermore, as oxLDL and acLDL are not identical ligands, certain scavenger receptors will exhibit varying affinities for each (Ottnad et al., 1995, Sparrow et al., 1989).

6 General Discussion

RA causes pain and swelling in the joints and is accompanied by a marked reduction in quality of life (Whalley et al., 1997); moreover, RA patients are at ~two-fold increased risk of developing CVD (Solomon et al., 2003, Turesson et al., 2003, Van Doornum et al., 2002). However, it is unknown how the underlying disease mechanism of RA influences the vascular environment.

The present study was principally carried out in human aortic adventitial tissue which was removed from the ascending aorta during the establishment of coronary artery bypass grafts. Blood vessels are known to exhibit different susceptibilities to atherosclerosis. Veins, for example, are usually less vulnerable than arteries. However, differences exist also within the arterial tree; for example the internal mammary artery (IMA), though of similar dimension to the coronary arteries, exhibits a ten-fold reduction in atherosclerosis risk (Ferro et al., 1990). This is in keeping with data presented in *chapter 3* which detected significantly less inflammation in the IMA than matched adventitial tissue.

The ascending aorta is a region with a known risk of atherosclerosis (Mills and Everson, 1991). Within the present study, all aortic specimens were taken from areas with minimal signs of atherosclerosis, a procedure necessary to reduce the risk of thromboembolic complication. However, as adventitial samples were removed from CVD patients requiring a bypass graft, this tissue provides a useful *ex vivo* model of aortic tissue that is at high risk of developing gross signs of atherosclerosis in the future. Furthermore, as samples were collected from CVD patients and patients with both CVD and RA, it was possible to investigate whether RA pathophysiology is capable of altering the adventitia in ways that may potentiate the onset of atherosclerosis.

Despite samples being removed from areas without gross clinical signs of atherosclerosis, the adventitia was found to possess an array of immunological properties. Inflammatory infiltration was detected in 18 of the 39 (46%) samples analysed (*section 3.2.3*). However, no significant difference in the extent of inflammatory infiltrate was observed between the CVD and RA+CVD cohorts. It remains to be elucidated what causes inflammatory infiltration into the adventitia, and whether adventitial infiltration underlies atherosclerosis pathogenesis. It would also be beneficial to characterise the IMA in more detail, with the aim of understanding why vascular inflammation is so rare in this tissue. As with many

conditions, understanding why certain tissue is at a reduced risk of disease often leads to breakthroughs in clinical treatment.

Despite no differences being detected in adventitial inflammation between the CVD and RA+CVD cohorts, it was found that TNFα expression was significantly greater in the RA+CVD cohort (*section 3.2.3*). Clearly, elevated adventitial TNFα in RA+CVD patients cannot be explained by an excess of inflammatory cells. However, further investigation revealed that serum TNFα expression is roughly ten times greater in the RA+CVD cohort compared to the CVD cohort. It is possible that the elevated levels of serum TNFα present in RA+CVD serum may infiltrate into, and reside in, the aortic adventitia. It remains to be investigated whether adventitial TNFα contributes to atherosclerosis, however it would appear likely, as several studies have shown TNFα to be associated with CVD and to posses pro-atherosclerotic properties (Ridker, 1997, McKellar et al., 2009).

It was also found that RA+CVD patients exhibited a trend towards greater HSP47 expression in the adventitia compared to CVD patients (*section 3.2.12*). HSP47 is a stress protein that acts as a molecular chaperone during intracellular processing of procollagen (Nakai et al., 1992), and a relationship between HSP47 expression and fibrosis has been previously reported (Rocnik et al., 2000).

As outlined in *section 1.5.4*, HSPs have been implicated as potential autoantigens in both RA and CVD (Pockley, 2002, Bodman-Smith et al., 2003). Furthermore, HSPs are immunodominant molecules, providing a principle epitope during pathogen recognition (Kaufmann, 1990) and often being implicated in human autoimmune disease. HSP expression is increased during exposure to cellular stress, a process which protects cells from the uncontrolled protein unfolding that often accompanies stress (Zuegel and Kaufmann, 1999). Although it remains to be determined, it is possible that environmental stress signals which accompany RA pathogenesis result in elevated adventitial HSP47 expression. This overexpression could potentiate atherosclerosis by providing a target for HSP47 specific autoantibodies. Alternatively, as it has previously been suggested that many of the HSPs have the property of damage-associated molecules (DAMPs), which are inducers of sterile inflammation and innate immunity (Kono and Rock, 2008), that HSP47 overexpression may facilitate the formation of a pro-inflammatory environment. The latter hypothesis would appear less likely when considering that it has already been shown that inflammation is not increased in the adventitia of RA+CVD patients.

The detection of inflammatory infiltrate, TNFα and the antimicrobial peptide calprotectin (*section* 3.*2.15*) in the aortic adventitia reinforced our previous hypothesis that bacteria may reside in the aortic adventitia and possibly contribute to atherosclerosis pathology. Furthermore, it is possible that the systemic inflammatory state which accompanies RA may alter patient susceptibility to certain bacterial species.

Bacterial DNA from a diverse range of species were detected in the aortic adventitia. Furthermore, this study demonstrated that RA+CVD patients had a significantly reduced bacterial heterogeneity in the adventitia when compared to CVD patients. Furthermore, *M. oryzae* was detected in all aortic adventitia samples from RA patients that were PCR-positive for the 16S rRNA gene, but in only 25% of PCR-positive samples from non-RA patients (*section 4.2.3*).

The detection of *M. oryzae* was somewhat surprising as this pink-pigmented, facultatively methylotrophic bacterium was first characterised following isolation from rice stem tissue in Korea (Madhaiyan et al., 2007). To the best of our knowledge, this is the first time that *M. oryzae* has been implicated in human disease. 16S rRNA gene sequencing analysis indicated that *M .oryzae* is most closely related to *M. mesophilicum*. Interestingly, *M. mesophilicum*, which is also a pink-pigmented, facultatively methylotrophic bacterium has been reported as a cause of opportunistic infections in immunocompromised hosts (Sanders et al., 2000) and has been isolated from several clinical sites, including blood, synovial, cerebral spinal fluid (CSF) (Kaye et al., 1992, Liu et al., 1997, Gilardi and Faur, 1984). Although the source of infection remains unclear, infection has been associated with environmental exposure. In particular, *M. mesophilicum* contamination of hospital tap water has been implicated as the source of an isolated nosocomial outbreak (Gilchrist et al., 1986).

In order to further characterise the potential atherogenic properties of *M. oryzae* we infected human primary macrophages. This study (*section 4.2.10*) highlighted that infection was characterised with an excessive inflammatory response, and subsequent analysis identified several pathways which could play a role in atherosclerosis. Curiously, granzyme B (encoded by *GZMB*), a serine protease that is highly expressed in cytotoxic T cells and natural killer cells, was ~200-fold up-regulated in macrophages following infection with *M. oryzae*. The principal function of granzymes is to induce the death of virus-infected and other transformed cells. Macrophages have previously been

demonstrated to not produce granzyme B, however it has recently been demonstrated that macrophages express granzyme B in the lesion areas of atherosclerosis and RA (Kim et al., 2007).

M. oryzae has previously been reported to be a Gram-negative bacterium (Madhaiyan et al., 2007). It was therefore very surprising when we determined that *M. oryzae* did not activate TLR4 signalling, but instead activated signalling from TLR2, the principle receptor for recognition of Gram-positive bacteria (*figures 4.17 and 4.18*). However, subsequent analysis confirmed that *M. oryzae* did not possess measurable levels of LPS or LOS (*figure 4.19*). Unfortunately, no images of the Gram stains performed on *M. oryzae* were available from the publication by Madhaiyan *et al*. (2007), so it is possible that owing to the inherent difficulty of Gram-staining certain bacillus species that staining was incorrectly interpreted. It is worth noting that although Pam3CSK4 was included in the assay depicted in *figure 4.18*, due to a technical oversight the hot phenol/water would not support extraction of lipopeptides from *M. oryzae*. Therefore, despite functional stimulation of TLR2 having been determined, it remains to be confirmed whether *M. oryzae* does indeed posses TLR2 ligand.

In a recent study, Canducci *et al.* (2012) characterised the antibody fragments produced by B-lymphocytes within the atherosclerotic lesions of four patients. Following molecular cloning, phage display and subsequent antibody generation it was demonstrated that all four patients possessed antibodies that cross-reacted with the outer membrane proteins (OMPS) of Gram-negative bacteria and with transgelin (TAGLN), a cytoskeleton protein expressed by smooth muscle cells (Canducci et al., 2012). Building upon this study, it would be beneficial to identify if antibodies raised against *M. oryzae* reside in the adventitia and whether these could similarly cross-react with self-antigens. It is possible that intermittent exposure to bacterial epitopes may lead to production of self cross-reacting antibodies that may persist following bacterial clearance.

Accumulating evidence suggests that alterations to the host microbiome precede RA and may play a key role in pathogenesis (Wu et al., 2010), however it is equally plausible that alteration to the microbiome follows RA and may lead to co-morbidity. Intriguingly, RA patients exhibit elevated levels of T_H17 cells and their signature cytokine IL-17 (*figure 5.1*) in the serum and although normal levels of circulating regulatory T-cells are expressed, they exhibit decreased functionality *ex vivo* (Valencia et al., 2006). Conceivably, altered T

cell functionality in parallel with the elevation of systemic pro-inflammatory mediators characteristic to RA supports a notion for RA driven dysbiosis and vulnerability to infection by a few 'select' organisms (e.g. *M. oryzae*). To what extent host immunity plays a role in aortic infection, and how the patterns of infection observed in this Norwegian population compare to others, remains to be elucidated.

The present study was limited by the necessity to characterise bacterial populations based on the sequencing of DNA which corresponded to the 16S rRNA gene. Ideally we would have expanded the value of these methods to generating a cDNA library and using 16S rRNA-targeted molecular biology tools to determine the transcriptional activity of bacterial populations. Although attempted, RNA was deemed not to be of sufficient quality to support cDNA synthesis. Owing to the inherent instability of RNA, it is likely that loss of quality was due to several freeze-thaw cycles that were incurred between surgery and experimentation. The correlation between cellular rRNA content and growth rate is a well-established concept (Poulsen et al., 1993), with rRNA transcription being approximately ten-fold elevated when the doubling time of *E. coli* decreases from 100 minutes to 24 minutes (Lu et al., 2009). 16S rRNA analysis of a cDNA library would therefore have allowed determination of whether bacteria in the adventitia were viable at the time of CABG surgery rather than the result of an historical infection. Furthermore, 16S rRNA cDNA analysis by quantitative PCR would allow the hypothesis that total bacterial burden may be altered in RA+CVD patients relative to CVD patients to be explored.

As well as analysing the adventitia of CVD and CVD+RA patients, the adventitia of CVD-PsA patients was also assessed for the presence of 16S rRNA. Interestingly no bacterial signatures were detected in this patient cohort. The symptoms of PsA closely resemble that of RA. However, it has been shown that the incidence rate of infection in PsA is similar to that of patients with psoriasis but not RA (Haddad et al., 2011), and the overall rate of infection is higher in RA patients compared to non-RA subjects (Doran et al., 2002). As both PsA and RA patients are generally prescribed a similar course of NSAIDs, DMARDs and biologics it would appear that unlike PsA, the pathophysiology of RA predisposes patients to infection. It remains to be determined whether the absence of bacterial DNA detection in the adventitia is due to PsA having a protective effect against infection.

In *chapter 5* the serum concentration of a broad range of cytokines, chemokines and growth factors was determined in RA, CVD, RA+CVD and healthy subjects. This study provided an opportunity to indentify biomarkers that were elevated in RA+CVD patients by a value greater than the sum of both RA and CVD patients. As highlighted and discussed in *section 5.3*, nine such biomarkers were identified (IL-1β, IL-2, IL-5, IL-13, IL-17, MIG, G-CSF, GM-CSF and IL-8). However, value was also attained from looking at the immune profile of the RA patients relative to healthy controls. Despite these patients not presenting clinical signs of CVD at the time of study, there is an increased likelihood that they will go on to develop CVD (Turesson et al., 2003). It is possible that the systemic immune profile of these patients could pre-dispose them to subsequent CVD. Although not possible due to time constraints in the current study, it would be beneficial to follow up the RA patients entered into this study and determine whether future CVD burden correlates with any of the biomarkers measured.

If considering the function of the biomarkers found to be elevated in RA+CVD and RA patients (*tables 5.2 and 5.3*) it is possible to speculate as to how such an immune profile may promote atherosclerosis. Endothelial dysfunction is considered to be an early step in atherosclerosis. In RA and RA+CVD there was a marked increase in the expression of TNFα and IL-1β, both of which are known to activate and induce migration and proliferation of endothelial cells and increase vascular permeability (Millauer et al., 1993, Inoue et al., 1998). Once activated, endothelial cells will support an infiltration of circulating monocytes/lymphocytes into the vessel wall. Owing to the elevated expression of MIP-1α, MIP-1β and IL-8, it is likely that the numbers of activated circulating monocytes and macrophages is already elevated in these patients. The concomitant presence of systemic IL-2 and IL-5 will support T- and B-cell expansion and differentiation, with the potential to generate antibodies against self (possibly HSP47) and bacterial antigens. Unless effective therapeutic intervention is achieved, monocytes and lymphocytes are likely to continue infiltrating into the vessel wall in an uncontrolled manner causing damage to the vessel wall and manifesting over time as an atherosclerotic lesion.

This project provides insight into the immunological activity and functional capacity of the aortic adventitia in atherosclerosis pathogenesis. The detection of increased TNFα, HSP47 and an altered bacterial profile in the adventitia of RA+CVD indicate that these important

factors may contribute to the increased CVD burden experienced in RA. These data have led us to propose a possible model of atherosclerosis pathogenesis in RA (*figure 6.1*). Furthermore, this study revealed that RA+CVD patients are characterised by an increased systemic pro-inflammatory phenotype, supporting a role for inflammation in accelerating comorbid CVD.

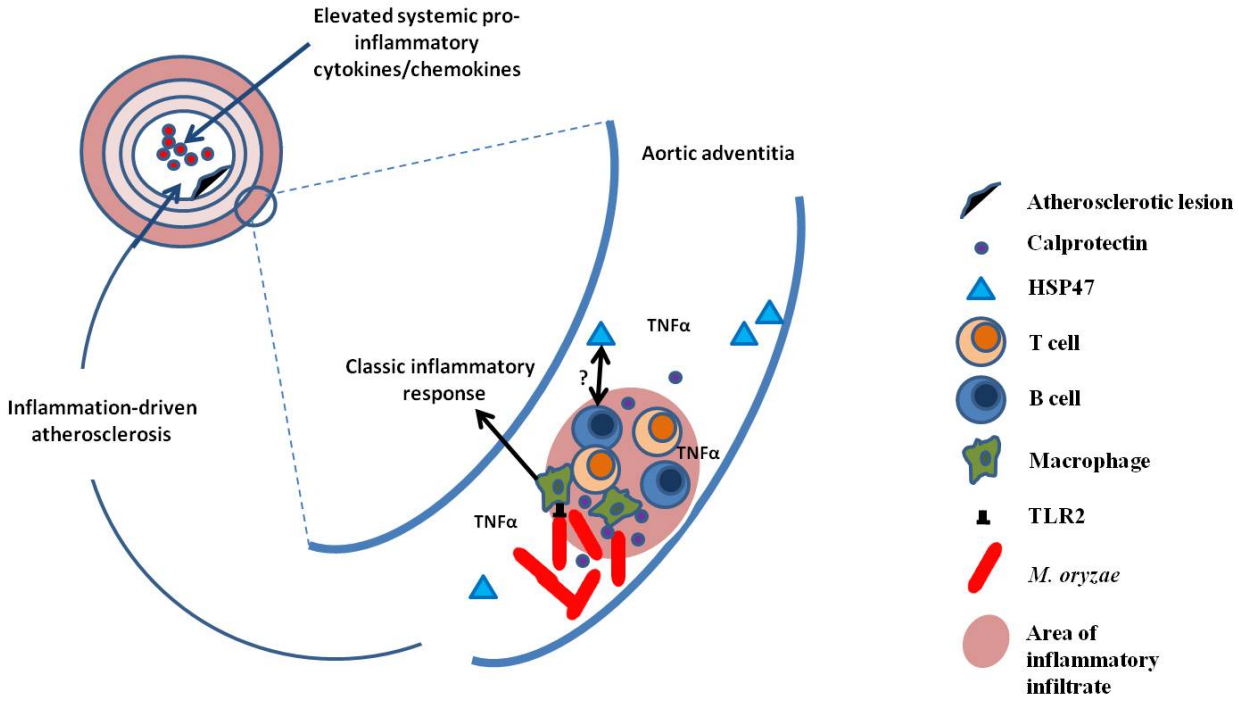

Figure 6.1 Model of adventitial-derived atherosclerosis in RA

A schematic model illustrating the possible effects of molecules detected in the aortic adventitia in atherosclerosis pathogenesis in RA patients. The altered systemic immune profile in RA may support *M. oryzae* infiltration into the adventitia. Bacteria interact with macrophages and other components of the adventitial inflammatory infiltrate to produce a classical pro-inflammatory response. This will further recruit inflammatory cells into the adventitia and lead to elevated expression of TNFα. B-cells may interact with elevated adventitial HSP47 leading to autoantibody generation and further perpetuate the inflammatory response. This molecular niche may accelerate atherosclerosis in RA and over time aid the formation of a characteristic lesion at the vessel wall.

Conclusions

This research was scoped to address three research questions (*section 1.6*). In conclusion it is necessary to summarise the principle findings.

- ***Is the aortic adventitia immunogenic? Does the inflammatory mileu of the aortic adventitia differ between CVD and RA+CVD patients?***

The aortic adventitia of CVD and CVD+RA patients has been confirmed as a site of considerable immunological interest, frequently exhibiting diffuse inflammatory infiltrates, the pro-inflammatory cytokine TNFα, the potential autoantigen HSP47 and the antimicrobial peptide calprotectin. The adventitia of RA+CVD patients was demonstrated to express significantly more TNFα than patients with CVD alone. These data may help explain why CVD is accelerated in RA patients and provides support for the involvement of the adventitia in atherosclerosis pathogenesis.

- ***Are bacteria present in the aortic adventitia, and do differences in the bacterial profile exist between CVD and RA+CVD patients?***

Four of 11 CVD and three of 11 RA+CVD aortic adventitia samples studied possessed bacterial DNA. The aortic adventitia of CVD+RA patients exhibited significantly lower bacterial heterogeneity. Interestingly *Methylobacterium orzyzae* was detected in all three RA+CVD samples positive for bacterial presence. *M. oryzae* was determined to be a mild TLR2 agonist and triggered a robust inflammatory response by macrophages. These data reinforce the hypothesis that bacteria may play an aetiological role in atherosclerosis, and suggest that the pathophysiology associated with RA predisposes aortic infection to a few select organisms.

- ***Does the systemic immune profile differ between RA, CVD and RA+CVD patients, and could any differences contribute to increased CVD burden in RA?***

RA patients exhibit an altered systemic immune profile compared to non-RA CVD patients and healthy controls. Furthermore, RA+CVD patients were shown to possess an altered systemic immune phenotype compared to both RA and CVD patients. Notably, IL-1β, IL-2, IL-5, IL-8, IL-13, IL-17, MIG, G-CSF and GM-CSF expression was greater in the serum of RA+CVD patients compared to the additive values of the RA and CVD patients. Systemic elevation of pro-inflammatory cytokines in RA patients likely contributes to atherosclerosis pathogenesis. Furthermore, GM-CSF was found to significantly decrease the rate at which human macrophages could endocytose oxLDL and form foam cells. When considered in isolation this finding suggests that the overexpression of GM-CSF observed in RA and RA+CVD patients may provide some atheroprotective benefit.

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Appendix

1. Universal primer for 16S sequencing

a) Bacterial 16S rDNA	Forward 5' CAGGCCTAACACATGCAAGTC 3' (63F)
(Annealing temp 60°C)	Reverse 5' GGGCGGWGTGTACAAGGC 3' (1387r).

2. Immunohistochemistry Reagents

Tris buffered saline (TBS)	8.76 g sodium chloride (150 mM), 6.05 g Tris base (50 mM) to 800 ml of distilled water. pH to 7.5 by addition of HCl. Make up to 1 litre by addition of distilled water. Add Tween-20 (Sigma-Aldrich) to 0.1% (v/v). Stored for up to 1 week at room temperature.
TBS + 0.05% Tween (TBST)	Add 200ml 10X TBS to 1800ml of deionised water followed by addition of 1ml Tween (Sigma-Aldrich, P5927) then mix
Trypsin working solution	0.5% Trypsin, 1% calcium chloride solution. Adjust pH to 7.8 by addition of 1M NaOH.
Citrate buffer	2.1 g of Citric acid (Sigma-Aldrich) dissolved in 800ml of distilled water. pH adjust to 6.0 by addition of 1 =M NaOH. Volune made up to 1 litre by addition of distilled water.
Hydrogen peroxide 0.5%	Prepare fresh for every use. Add 5ml of 30% hydrogen peroxide (H_2O_2) to 295ml of methanol (both supplied by Sigma-Aldrich)
Impact DAB	1ml diluent, 1 drop chromagen, mex (Vector Laboratories)
Secondary Antibody	Biotinylated anti-mouse IgG (produced in horse) (Vector Laboratories) (1.5mg/ml)

Vacstatin *Elite* ABC kit

Avidin and **B**iotinylated horseradish peroxidise macromolecular **C**omplex (Vector Laboratories, PK6100)

	To 5ml TBST:	2 drops of reagent A, mix
		2 drops of reagent B, mix
	Incubate at room temperature for 30 minutes before use	
Wax pens	Immedge pen (Vector Laboratories, H-4000)	
Blocking serum	2.5% normal horse serum (Vector Laboratories, S-2000) in TBST (unless otherwise stated)	
Negative control	Mouse IgG2A, (500µg/ml) (R&D, MAB003)	
	Mouse IgG1, (500µg/ml) (R&D, MAB002)	
FACS buffer	3% BSA 0.05% NaN_3 in 1 x PBS	

3. Lipoprotein isolation/visulisation reagents

a) Copper reagent

20g sodium carbonate dissolved in 260ml H_2O, 0.4g cupric sulphate pentahydrate dissolved in 20ml H_2O and 0.2g sodium potassium tartrate dissolved in 20ml H_2O. The resulting solutions are then combined.

b) Barbital buffer

18.2g (5, 5-Diethylbarbituric Acid), 10mmol/L reconstituted; 5, 5-Diethylbarbituric Acid sodium salt 50mmol/L

c) Oil-red-O stock solution

0.5g of Oil-red-O powder was dissolved in 100ml isopropanol. This was performed overnight with gentle agitation.

d) Oil-red-O working solution

Mix 6ml of Oil-red-O stock solution with 4ml of ddH_2O. Allow to sit at room temperature for 20 minutes and then filter (0.2μm).

4. Primers for quantitative PCR

Human GAPDH	Forward 5' ACA GTC AGC CGC ATC TTC TT 3'
(Annealing Temp: 55^{o}C)	Reverse 5' AAA TGA GCC CCA GCC TTC T 3'
Human SRA-1	Forward 5' GAC TTT GGT TCC CGT GTT GT 3'
(Annealing Temp: 55.6^{o}C)	Reverse 5' GGA GAG AGG TGA TGG TGG AG 3'
Human CD36	Forward 5' CAA GGA AAA TGT AAC CCA GGA C 3'
(Annealing Temp: 56.4^{o}C)	Reverse 5' GCC ACA GCC AGA TTG AGA AC 3'
Human SRB1	Forward 5' CCC TAA CCA GGA GGC ACA C 3'
(Annealing Temp: 56.9^{o}C)	Reverse 5' CAG GAC CAC AGG CTC AAT CT 3'
Human MARCO	Forward 5' TCT GGG GAG CAA GGA GTA AA 3'
(Annealing Temp: 58.1^{o}C)	Reverse 5' ATT GTC CCC CAG GTA CCA CT 3'

5 *M. oryzae* and *M. mesophilicum* sequence alignment

Top line: *Methylobacterium oryzae*
Bottom line: Methylobacterium mesophilicum

Length: 1023
Identity: 973/1023 (95.1%)
Similarity: 973/1023 (95.1%)
Gaps: 11/1023 (1.1%)

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EMBOSS_001         1 ctaaccgtggtcagcggcggacgggtgagtaacgcgtgggaacgtgcctt     50
                     ||.  ||..|||||||||||||||||||||||||||||||||||||||.|
EMBOSS_001         1 ctt--cggtgtcagcggcggacgggtgagtaacgcgtgggaacgtgccct     48

EMBOSS_001        51 ccggttcggaataaccctgggaaactagggctaataccggatacgccctt    100
                     |.|||||||||||||.|.||||||||.|.|||||||||||||||||||||
EMBOSS_001        49 ctggttcggaataactcagggaaacttgagctaataccggatacgccctt     98

EMBOSS_001       101 atggggaaaggtttactgccggaagatcggcccgcgtctgattagctagt    150
                     .||||||||||.||.||||||||.|||||||||||||||||||||||||||
EMBOSS_001        99 ttggggaaaggcttgctgccggaggatcggcccgcgtctgattagctagt    148

EMBOSS_001       151 tggtggggtaacggcctaccaaggcgacgatcagtagctggtctgnnaga    200
                     |||||.|||||||||..|||||||||||||||||||||||||||  |||
EMBOSS_001       149 tggtgaggtaacggctcaccaaggcgacgatcagtagctggtctg--aga    196

EMBOSS_001       201 ggaagatcaagccacactgcgcactgaggacacggcccagactcctacgg    250
                     |||.|||| |||||||||| |.||||| ||||||||||||||||||||||
EMBOSS_001       197 ggatgatc-agccacactg-ggactga-gacacggcccagactcctacgg    243

EMBOSS_001       251 gnaagcaacagtggagaatattagacaatgcgcgcaagcctgatccagcc    300
                     | |.|||.||||||.|||||||.|||||||.||||||||||||||||||||
EMBOSS_001       244 g-aggcagcagtggggaatattggacaatgggcgcaagcctgatccagcc    292

EMBOSS_001       301 atgcctgagggagtgatgaaggccttagggttgttaaagctcttttatcc    350
                     ||||| |.|.|||||||||||||||||||| ||||||||||||||||||||
EMBOSS_001       293 atgcc-gcgtgagtgatgaaggccttagggttg-taaagctcttttatcc    340

EMBOSS_001       351 gggacgataatgacggtaccggacgaataagccccggctaactccgtccc    400
                     ||||||||||||||||||||||.||||||||||||||||.|||.||
EMBOSS_001       341 gggacgataatgacggtaccggaggaataagccccggctaacttcgtgcc    390

EMBOSS_001       401 agcagccgcggtaatacgaaggggctagcgttgctcggaatcactggcc    450
                     ||||||||||||||||||||||||||||||||||||||||||||||||.|
EMBOSS_001       391 agcagccgcggtaatacgaaggggctagcgttgctcggaatcactgggc    440

EMBOSS_001       451 gtaaagggcgcgtaggcggcgttttaagtcgggggtgaaagcctgtggct    500
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       441 gtaaagggcgcgtaggcggcgttttaagtcgggggtgaaagcctgtggct    490

EMBOSS_001       501 caaccacagaatggccttcgatactgggacgcttgagtatggtagaggtt    550
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EMBOSS_001       491 caaccacagaatggccttcgatactgggacgcttgagtatggtagaggtt    540

EMBOSS_001       551 ggtggaactgcgagtgtagaggtgaaattagtagatattcgcaagaacac    600
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EMBOSS_001       541 ggtggaactgcgagtgtagaggtgaaattcgtagatattcgcaagaacac    590

EMBOSS_001       601 cggtggcgaaggcggccaactggaccattactgacgctgaggcgcgaaag    650
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EMBOSS_001       591 cggtggcgaaggcggccaactggaccattactgacgctgaggcgcgaaag    640

EMBOSS_001       651 cgtggggagcaaacaggattagataccctggtagtccacgccgtaaacga    700
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EMBOSS_001       641 cgtggggagcaaacaggattagataccctggtagtccacgccgtaaacga    690
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EMBOSS_001       701 tgaatgccagctgttggggtgcttgcaccgcagtagcgcagctaacgctt    750
                     ||||||||||||||||||||||||||||.||||||||||||||||||||
EMBOSS_001       691 tgaatgccagctgttggggtgcttgcacctcagtagcgcagctaacgctt    740

EMBOSS_001       751 tgagcattccgcctggggagtacggtcgcaagattaaaactcaaaggaat    800
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       741 tgagcattccgcctggggagtacggtcgcaagattaaaactcaaaggaat    790

EMBOSS_001       801 tgacgggggcccgcacaagcggtggagcatgtggtttaattcgaagcaac    850
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EMBOSS_001       791 tgacgggggcccgcacaagcggtggagcatgtggtttaattcgaagcaac    840

EMBOSS_001       851 gcgcagaaccttaccatcctttgacatggcgtgttacccagagagatttg    900
                     |||||| ||||||||||||||||||||||||||....||||||||..
EMBOSS_001       841 gcgcag-accttaccatcctttgacatggcgtgttatggggagagattcc    889

EMBOSS_001       901 gggtccacttcggtggcgcgcacacaggtgctgcatggctgtcgtcagct    950
                     ..||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       890 cagtccacttcggtggcgcgcacacaggtgctgcatggctgtcgtcagct    939

EMBOSS_001       951 cgtgtcgtgagatgttgggttaagtcccgcaacgagcgcaacccacgtcc   1000
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       940 cgtgtcgtgagatgttgggttaagtcccgcaacgagcgcaacccacgtcc    989

EMBOSS_001      1001 ttagttgccatcattcagttggg   1023
                     |||||||||||||||||||||||
EMBOSS_001       990 ttagttgccatcattcagttggg   1012
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6 Genes analysed by TLDA

Gene	Name	Class	Functions
IL-1a	Interleukin 1 alpha	Cytokine	Produced by macrophages and neutrophils with a central role in immune regulation
IL-1b	Interleukin 1 beta	Cytokine	Produced by macrophages and regulates inflammation, cell proliferation, differentiation and apoptosis
IL-2	Interleukin 2	Cytokine	Stimulates proliferation of T and B cells
IL-4	Interleukin 4	Cytokine	Induces differentiation of naive helper T cells to Th2 cells
IL-5	Interleukin 5	Cytokine	Stimulates B cell growth and mediates eosinophil activation
IL-6	Interleukin 6	Cytokine	Pro-inflammatory pleiotropic cytokine
IL-7	Interleukin 7	Cytokine	Stimulates proliferation of B, T and NK cells
IL-9	Interleukin 9	Cytokine	Produced by $CD4^+$ cells and stimulates cell proliferation and inhibits apoptosis
IL-10	Interleukin 10	Cytokine	Anti-inflammatory/resolving
IL-12a	Interleukin 12 alpha	Cytokine	Differentiation of Th1 and Th2 cells and stimulates IFNγ production by PBMCs
IL-12b	Interleukin 12 beta	Cytokine	Differentiation of Th1 and Th2 cells and stimulates IFNγ production by PBMCs
IL-13	Interleukin 13	Cytokine	Inhibits inflammatory cytokine production
IL-15	Interleukin 15	Cytokine	Stimulates proliferation of T-lymphocytes
IL-17	Interleukin 17	Cytokine	Induces stromal cells to produce proinflammatory and hematopoietic cytokines
Il18	Interleukin 18	Cytokine	Pro inflammatory cytokine produced by macrophages
TNF	Tumour necrosis factor	Cytokine	Regulation of a wide spectrum of biological processes including cell proliferation, differentiation, lipid metabolism and apoptosis.
IFN γ	Interferon gamma	Cytokine	Produced by macrophages and T cells and has antiviral and antibacterial activities
Tgfb1	Transforming growth factor beta 1	Cytokine	Regulates cell growth, cell proliferation, cell differentiation and apoptosis

Table 6.1 List of human and murine cytokines examined by TLDA

Gene	Name	Class	Functions
Cxcl10	Chemokine (C-X-C-motif) ligand 10	Chemokine	Secreted by monocytes, endothelial cells and fibroblasts in reponse to IFN γ . Chemoattractant for macrophages, T cells NK cell
Cxcl11	Chemokine (C-X-C motif) ligand 11	Chemokine	Chemotactic for activated T cells and is highly produced in peripheral blood leukocytes
Ccl2	Chemokine (C-C motif) ligand 2	Chemokine	Recruits monocytes, memory T cells and dendritic cells to sites of infection and inflammation
Ccl3	Chemokine (C-C motif) ligand 3	Chemokine	Involved in the acute inflammatory state for the recruitment and activation of polymorphonuclear leukocytes
Ccl5	Chemokine (C-C motif) ligand 5	Chemokine	Promotion of inflammatory infiltrate and T-cell chemotaxis
Ccl19	Chemokine (C-C motif) ligand 19	Chemokine	Chemoattractant for dendritic cells, antigen engaged B cells and $CCR7^{+}$ memory T cells.
Ccr2	Chemokine (C-C motif) receptor 2	Chemokine	Receptor for Ccl2
Ccr4	Chemokine (C-C motif) receptor 4	Chemokine	Receptor for Ccl2, Ccl17, Ccl22
Ccr7	Chemokine (C-C motif) receptor 7	Chemokine	Receptor for Ccl19, Ccl21
Cxcr3	Chemokine (C-X-C motif) receptor 3	Chemokine	Receptor for Cxcl10, Cxcl11
Il8 *(Human)*	Interleukin 8	Chemokine	Functions as a chemoattractant and is a potent angiogenic factor

Table 6.2 List of human and murine chemokines examined by TLDA. Species specific genes are shown in bold.

Gene	Surface lineage
CD19	B lymphocytes
CD28	T cells that provide co-stimulatory signals
CD34	hematopoietic cells
CD38	T cells, B cells, NK cells and plasma cells
CD3e	T cells
CD4	T helper cells
CD68	Macrophages
CD80	B cells and monocytes that provides a costimulatory signal necessary for T cell activation and survival
CD86	Antigen presenting cells
CD8a	Cytotoxic T cell
Ctla4	T cell
Icos	Activated T cells

Table 6.3 List of human and murine surface lineage markers examined by TLDA

Gene	Name	Class	Functions
18S	18S ribosomal RNA	Housekeeping	18S rRNA is part a component of the ribosomal subunit
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase	Housekeeping	Breaks down glucose during metabolism

Table 6.4 List of human and murine housekeeping genes examined by TLDA

Gene	Name	Class	Functions
Col4a5	Collagen alpha-5(IV) chain	Structural protein	Encodes one of six subunits of type IV collagen, the main component of connective tissue
Actb	Beta-actin	Structural	Nonmuscle cytoskeletal actin involved in motility, structure and integrity
Nos2	Nitric oxide synthase 2	Enzyme	Synthesises the biological mediator nitric oxide which has antimicrobial activity
Cyp1a2	Cytochrome P450 1A2	Enzyme	A member of the mixed function oxidase system involved in xenobiotics metabolism
Cyp7a1	Cholesterol 7 alpha hydroxylase	Enzyme	Oxidises cholesterol using molecular oxygen
Gusb	Beta-glucuronidase	Enzyme	Hydrolase that degrades glycosaminoglycans
Hmox1	Heme oxygenase 1	Enzyme	Essential enzyme in heme catabolism
Ece1	Endothelin converting enzyme 1	Enzyme	Proteolytic processing of EDN1,2,3 to biologically active peptides
Edn1	Endothelin 1	Peptide	Vasoconstricting peptide having a key role in vascular homeostatsis
Hprt1 *(Murine)*	Hypoxanthine-guanine	Enzyme	Plays a central role in generation of purine nucleotides through the purine salvage patheway
Pgk1	Phosphoglycerate kinase 1	Enzyme	Glycolytic enzyme that catalyses conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate
Ptgs2	Prostaglandin-endoperoxide synthase 2	Enzyme	Converts arachidonic acid to prostaglandin endoperoxide H2 during inflammation
Lrp2	Low density lipoprotein-related protein 1	Receptor	Mediates endocytosis of ligands leading to degradation in lysosomes or transcytosis
Tfrc	Transferrin receptor 1	Protein	Iron delivery from transferring to cells
Ski	Ski proto-oncogene	Protein	Negatively regulates TGF-beta
Fn1	Fibronectin	Glycoprotein	Glycoprotein of the extracellular matrix that binds integrins
Gnly *(Human)*	Granulysin	Protein	Present in cytotoxic granules of cytotoxic T cells, macrophages and natural killer cells and has antimicrobial activity
Ace *(Human)*	Angiotensin I converting enzyme	Enzyme	Converts angiotensin I to Angiotensin II and is also able to inactivate bradykinin
Ren	Renin	Enzyme	Activates the rennin-angiotensin system which mediates extracellular volume

Table 6.5 List of human and murine genes encoding intracellular proteins and enzymes examined by TLDA. Species specific genes are shown in bold

Gene	Name	Class	Functions
Socs 2 *(Murine)*	Suppressor of cytokine signalling 2	Transcription factor	Regulation of IGF1R
Smad3	SMAD family member 3	Transcription factor	Transcriptional modulator activated by TGFb
Smad7	SMAD family member 7	Transcription factor	Antagonistic and locks activation of SMADs
Tbx21	T-box transcription factor TBX21	Transcription factor	Controls expression of IFNG in Th1 and natural killer cells
Ikbkb	Inhibitor of kappa light polypeptide gene	Signalling factor	Phosphorylates inhibitors of NF-kappa-B thus leading to the dissociation of inhibitor/NF-kappa-B complex
Stat1 *(Murine)*	Signal transducer and activator of transcription 1	Signalling factor	Upregulates genes in response to type I, II or III interferons
Stat3	Signal transducer and activator of transcription 3	Signalling factor	Mediates genes that play key roles in cell growth and apoptosis
Stat4 (Murine)	Signal transducer and activator of transcription 4	Signalling factor	Mediates responses to IL12 in lymphocytes
Stat6 *(Murine)*	Signal transducer and activator of transcription 6	Signalling factor	Exerts IL4 mediated biological responses and induces expression of BCL2L1
Nfkb1 *(Murine)*	Nuclear factor NF-kappa-B p105 subunit	Transcription factor	Involved in many biological functions such as inflammation, immunity, differentiation and cell growth
Nfkb2	Nuclear factor NF-k-B p49/p100 subunit	Transcription factor	Involved in cellular responses to stimuli such as cytokines and stress and plays a key role in regulating the immune response to infection

Table 6.6 List of human and murine genes encoding transcription factors and signalling factors examined by TLDA. Species specific genes are shown in bold.

Gene	Name	Class	Functions
Bax	Bcl2-associated X protein	Pro-apoptotic	Promotes apoptosis by competing with Bcl2
Fas	FAS receptor	Pro-apoptotic	Death receptor on the cell surface which lead to programmed cell death
Faslg	FAS ligand	Pro-apoptotic	Ligand which binds the FAS receptor
Bcl2	B cell lymphoma 2	Pro-survival	Suppresses apoptosis in a variety of cell systems
Bcl2l1	Bcl2-like 1	Pro-survival	Inhibitor of cell death. Inhibits activation of caspases.
Csf1	Macrophage colony stimulating factor	Growth factor	Influences hematopoietic stem cells to differentiate into macrophages
Csf2	Granulocyte macrophage colony stimulating factor	Growth factor	Stimulates stem cells to produce granulocytes and monocytes
Csf3	Colony stimulating factor 3	Growth factor	Controls the production, differentiation and function of granulocytes
Vegfa	Vascular endothelial growth factor A	Growth factor	Specifically acts on endothelial cells with effects including increased vascular permeability, inducing angiogenesis and inhibiting apoptosis
C3	Complement component 3	Immune effector	Plays a central role in the complement system and contributes to innate immunity
Prf1	Perforin	Immune effector	A cytolytic protein found in granules of T cells and NK cells. Upon degranulation, perforin inserts into the target cell's membrane forming a pore.
Gzmb	Granzyme B	Immune effector	Expressed by cytotoxic T cells and NK cells and induces rapid induction of target cell apoptosis
Lta	Lymphotoxin-alpha	Immune effector	Mediates a large variety of inflammatory, immunostimulatory and antiviral responses and is also involved in the formation of secondary lymphoid organs
Agtr1 ***(Human)***	Angiotensin II receptor type 1	Pro- apoptotic receptor	Mediates programmed cell death
Agtr2	Angiotensin II receptor type 2	Pro- apoptotic receptor	Mediates programmed cell death

Table 6.7 List of human and murine growth factors, immune effector and pro/anti-apoptotic genes examined by TLDA. Species specific genes are shown in bold.

Gene	Name	Class	Functions
B2m *(Mruine)*	Beta-2 microglobulin	Surface protein	Component of MHC class I molecules and is involved in endocytosis regulation
H2-ea *(Murine)*	Histocompatibility 2, class II antigen	Cell surface protein	Immune response antigen that function in T cell dependent immune response
Tnfrsf18	Tumour necrosis factor receptor superfamily 18	Cell surface recpetor	Key role in regulating immunological self tolerance
Cd40	CD40 molecule, TNF receptor superfamily 5	Cell surface recpetor	CD40 activates antigen presenting cells
Cd40lg	Cd40 ligand	Cell surface ligand	Primarily expressed on activated T cells and binds to CD40 on antigen presenting cells
Sele	E-selectin	Cell adhesion	Expressed only on activated endothelial cells and plays a role in leukocyte recruitment
Selp	P-selectin	Cell adhesion	Expressed on the surface of activated endothelial cells and functions in leukocyte recruitment
Ptprc	Protein tyrosine phosphatise receptor C	Enzyme	Signalling molecule with extracellular domain. Regulates a variety of cellular processes including growth and differentiation.
Hla-dra *(Human)*	HLA class II histocompatability antigen, DR alpha chain	Cell surface protein	Plays a central role in the immune system by presenting peptides derived from extracellular proteins
Hla-drb1 *(Human)*	HLA class II histocompatability antigen, DRB1-9 beta	Cell surface protein	Plays a central role in the immune system by presenting peptides derived from extracellular proteins
Myh6 *(Human)*	Myosin-6	Motor protein	Involvement in a wide range of motility processes
Il2-RA *(Human)*	Interleukin-2-receptor alpha chain	Surface receptor	High affinity IL-2 receptor
Icam1 *(Human)*	Intercellular adhesion molecule 1	Surface glycoprotein	Supports binding of leukocytes

Table 6.8 List of human and murine cell surface proteins and adhesion molecules examined by TLDA. Species specific genes are shown in bold